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The invention relates to a biochemical assay for wide class of hydrophobic Coenzyme A esters wherein the analyte is caused to react with a specifically binding, modified protein, and thereby causing a detectable signal. A one step assay for hydrophobic CoA esters in whole blood, serum, food and feed preparations, tissue extracts, acyl-CoA synthetase reaction media and various laboratory conditions using a modified Coenzyme A- and acyl-CoA binding protein (ACBP) is provided. Furthermore the invention relates to a construct comprising a peptide and a signal moiety for performing an assay, a kit for assaying hydrophobic CoA esters and a nucleotide sequence encoding the peptide as well as an expression vector and a cell comprising the nucleotide sequence.

Background of the Invention

An obligatory step in beta-oxidation, incorporation in to complex lipids or modification of fatty acids in living cells is conversion to its Coenzyme A thioester derivative (acyl-CoA). Besides playing a key role in lipid metabolism acyl-CoA esters have also been shown to act as regulatory molecules regulating enzyme activities, vesicular transport, hormone signalling, Ca^{2+} flux, ionchannels and the rate of transcription of specific genes (Færgeman & Knudsen, 1997; Biocem. J 323, p 1-12).

Long chain free fatty acid (FFA) with acyl chains > 16 carbons are quantitatively the most important physiological energy source. The concentration of FFA in growth media and circulating blood is the rate determining factor in regulation of fatty acid uptake (Glatz and van der Vusse, 1996; 35, 243-282) and have been shown to affect intracellular acyl-CoA concentrations (Sterchele, et. al.1994; Biochem. Pharm.48, 955-966). Although fatty acids and acyl-CoA esters are important and essential for normal physiological function they are also potent modulators of cellular activity (Færgeman & Knudsen,1997; Biocem.J 323, 1-12). Dietary fatty acids, through their influence on circulating fatty acid and intracellular acyl-CoA levels and composition, specifically modulate the onset of various diseases including cancer (Cave W.T., 1991; FEBS2166; Welsch C.W. 1992; Cancer Res. Suppl. 52, 2040-2048), atherogenesis (Chin, J.P., 1994; Prost. Leuk, Essent. Fatty Acids, 50, 211-

222), hyperlipidemia (Grundy and Denke, 1990; J. Lipid. Res. 31, 1149-1172), insulin resistance (Storlien, L.H., 1987; Science 237, 885-888) and hypertension (Moris, et. al., 1993; Circulation 88, 523-533).

In many instances determination of total fatty acids levels is of significant importance in diagnosis and treatment of disease or studying the mechanisms causing it. For example fatty acids are believed to be an important factor in the cause of ventricular arrhythmiaes during acute myocardial infarction (Makiguchi M, et. al. (Japan) Jul 1988, 63, 624-634). Differences in circulating levels of fatty acid are found in AIDS patients (Christeff, et. al., 1988 Eur.J. Cancer. Clin. Oncol. 24, 1179-1183). Plasma fatty acid concentrations in non insulin dependent diabetes mellitus are believed to be indicative for insulin resistance (Fraze et. al. 1985; J. Clin. Endocrinol. Metab. 61, 807-811). Fatty acid have been implicated in pathogenesis of thromboatherosclerosis, (Travella et al, 1985, Nutr. Res. 5, 365-65). Elevated levels of fatty acids have been found in human cancer patients and animal models (Storlien, L.H., 1987; Science 237, 885-888).

Because the circulating level of FFA influences the intracellular level of acyl-CoA esters, these esters could play an essential role in mediating regulatory and pathogenic effects of increased circulating FFA in various diseases mentioned above.

These effects could involve regulation of acetyl-CoA carboxylase, AMP-activated kinase-kinase, mitochondrial acyl-CoA synthetase, citrate transporter, HMG-CoA reductase, carnitin palmitoyl-CoA transferase, long-chain acyl-CoA dehydrogenase, hormone sensitive lipase, adenine nucleotide translocase, glucokinase, glucose-6-phosphate dehydrogenase, glucocose-6-phosphatase, pyruvate dehydrogenase, Ca^{2+} release from and uptake in intracellular stores, sodium/potassium ATPase, ATP sensitive potassium channels, protein kinase C, nuclear thyroid hormone receptor, vesicular transport, and proteolysis (see Færgeman and Knudsen, Biochem J. 323, 1-12 1997 for review)

Long chain acyl-CoA esters are highly amphiphatic molecules, which bind unspecifically to proteins, test tube walls and they partition into lipid membranes ($k = 1,5 \times 10^5$, Færgeman and Knudsen, 1997, Biochem J., 323, 1-12). The concentration

of free unbound acyl-CoA esters which is the regulatory species is therefore very different from the total concentration. Measurement of free long chain acyl-CoA *in vivo* and *in vitro* therefore has important applications in a wide variety of biochemical, biophysical, cell biologic and physiological research. Various HPLC, GC and enzymatic methods for determination of total acyl-CoA levels in tissue extracts and body fluids have been developed. However, no method is yet available for determining free acyl-CoA levels in tissue extracts, body fluids or in cytosol of living cells (Bækdal et. al. 1996; Advances in Lipid Methodology - Three, 109-131, Editor, Christie W.W., The Olie Press, Dundee Scotland, UK, for review).

A number of methods for determination of total fatty acid in blood, body fluids, cell culture media have been developed. One set of methods require extraction by organic solvent essentially as described by Folch et. al., 1957 (J. Biol. Chem. 226, p 497). The extracted fatty acids are subsequently quantified by gas-chromatography after methylation (Baty, and Pazouki, 1987 Chromatography, 395, p 403), by complexing to 63Ni in the organic phase in a two phase system (Ilo, R.J., 1970, Anal. Biochem. 36, p105) or by HPLC after derivatisation with a UV absorbing group (Miwa et. al., 1987, J. Chromatography 416, p 237). In another method (US patent no. 4,491,631) fatty acids are converted to acyl-CoA esters by acyl-CoA synthetase and quantified in an enzyme linked acyl-CoA dehydrogenase assay. This assay has recently been marketed in a different version where the acyl-CoA formed by the acyl-CoA synthetase is oxidised by acyl-CoA oxidase and the formed H_2O_2 is quantified by reaction with 3-methyl-N-ethyl-N-(beta-hydroxyethyl)-aniline to give a dye (Wako Chemicals USA, Inc.Richmont, VA 23237, USA).

Very recently, a method for determination of free fatty acids in blood using fluorescently modified fatty acid binding protein (ADIFAB) has been developed (Richieri et al, J. Biol. Chem.267, 23495-23501, 1992, US patent 5,470,714). This method can also be used to calculate total circulating total fatty acid concentration if the serum albumin concentration and its binding properties are know (Richieri et al, Molecular and Cellular Biochemistry, 192: 87-94, 1999). A disadvantage of this assay is that the binding between the analyte and the sensor is not very strong. The dissociation constant, K_D, for the complex between ADIFAB and various common fatty acids (palmitate, oleate, linileate, arachidonate, linolenate) lies in the range of 0.28 to 2.5 mM. In the presence of albumin (which is present in all blood samples) in

the sample, the fatty acids bind to both ADIFAB and albumin. Thus, in order to make a reliable estimate of the concentration of free fatty acid, the concentration of albumin in the sample must also be known. Furthermore, fatty acids have a high affinity to surfaces, especially to plastic surfaces. In an assay based on fatty acid binding protein, both the protein, albumin and any plastic surfaces will compete for the free fatty acids with approximately the same affinity resulting in rather unpredictable results.

In US 5,512,429 (BRITISH TECHNOLOGY GROUP LTD.) another method for selectively measuring fatty acids using a probe is disclosed. The disclosure more specifically concerns a method for assaying an enzyme being capable of releasing fatty acids from a substrate or for assaying fatty acids. According to the method described, serum albumin is first removed from the sample (which is most often a serum sample). The enzyme activity or the concentration of the fatty acid is measured by measuring the binding of the fatty acids to a fatty acid binding protein. According to the disclosure the binding should be a binding with a dissociation constant of 10^{-5}M or less. The method for detecting the fatty acid-FABP binding is by a competition assay with a known amount of a radioactively labelled fatty acid. In a particularly preferred embodiment, the label is a polycyclic fluorophore, especially a naphtalene or anthracene having a polarity-sensitive fluorescent group. As the label moves from a polar to a non-polar environment, the fluorescent group undergoes a change in fluorescent emission.

In a later publication by the same authors (US 5,449,607 (BRITISH TECHNOLOGY GROUP INC.)) it is asserted that there is no need for removal of albumin prior to performing the assay due to the high specificity of the binding. This may be possible by standardisation of the amount of albumin in the samples and the calibration samples. Under all circumstances it is inevitable that albumin competes with FABP for the free fatty acids and that albumin thus binds at least a fraction of the free fatty acids in the sample.

In general, the prior art methods for measuring the concentration of fatty acids and related compounds through binding assays are characterised by low precision due to the relatively low affinity for fatty acids and low selectivity, since fatty acid binding

proteins have a considerable affinity towards almost any hydrophobic compound of a certain size.

Summary of the invention

A first aspect of the invention relates to a method for determination of the concentration of free unbound hydrophobic Coenzyme A ester in a sample comprising the steps of

- providing a hydrophobic Coenzyme A binding construct exhibiting a first signal when unbound and exhibiting a measurably different second signal when bound to a hydrophobic Coenzyme A ester,
- contacting the sample with the labelled hydrophobic Coenzyme A binding construct,
- allowing at least one species of unbound free hydrophobic Coenzyme A ester to bind to the hydrophobic Coenzyme A binding construct forming a complex comprising a hydrophobic Coenzyme A ester and the hydrophobic Coenzyme A binding construct,
- detecting a signal from the complex,
- correlating the signal to the concentration of the at least one species of hydrophobic Coenzyme A ester in the sample.

The method according to the present invention provides an easy, rapid and yet highly specific and accurate method for measuring the concentration of hydrophobic Coenzyme A esters.

All compounds that can be converted to hydrophobic CoA esters can be measured indirectly by the method according to the invention by combination with suitable reactions for hydrolysis and Coenzyme A thioesterification. Such compounds include but are not limited to free fatty acids, lipids, triacylglycerides, phospholipids, cholestrolesters.

A great advantage of the method according to the present invention is the high affinity of the peptide comprised in the construct for hydrophobic CoA esters. The K_D of the construct with respect to hydrophobic CoA esters is preferably several orders of magnitude lower than the affinity of prior art constructs used for binding of fatty

acids. Due to the increased binding affinity the interference of other potential sinks for hydrophobic CoA esters such as albumin, Fatty Acid Binding Proteins or plastic surfaces with the binding assay is markedly reduced. The result is a much more precise estimation of the concentration of the hydrophobic CoA esters than hitherto possible.

Another advantage of the method according to the present invention is that the peptide part of the construct is extremely selective in its binding and binds in reality only hydrophobic CoA esters. The binding affinity of the constructs for other hydrophobic compounds such as free fatty acids, is extremely low and the presence of other hydrophobic compounds in the sample thus does not interfere with the assay according to the invention. Thus there is normally no requirement for purification or fractionation of the sample or isolation of analytes prior to performing the method.

In a further aspect, the invention relates to a construct for binding hydrophobic Coenzyme A ester comprising a heterologous peptide capable of binding at least one species of hydrophobic Coenzyme A ester, and a signal moiety.

Due to the high specificity and high affinity of these constructs towards hydrophobic CoA esters they are especially suited for use in the method for determination of the concentration of the hydrophobic compounds mentioned above.

The use of the constructs presented herein represents a unique way to measure free acyl-CoA concentrations of physiological important, highly amphiphatic, medium and long chain acyl-CoA esters. Long-chain acyl-CoA esters partition into membranes, stick to proteins and test tube walls. All previously published methods for measurement of acyl-CoA measure total acyl-CoA concentration including the very small fraction of free acyl-CoA, which is the biologically active fraction. This very small fraction can only be measured with the constructs according to the present invention. From the literature it is clear that knowledge of the free acyl-CoA concentration *in vivo* and *in vitro* conditions is the key to understand the function of these very important molecules in regulation of key cell functions including gene expression (Faergeman and Knudsen, 1997; Biochem J. 323, 1-12). One advantage with the present constructs is their high degree of specificity for hydrophobic-CoA

esters only. The CoA head group determines the binding specificity of ACBP by interacting with specific amino acid residues in the binding site and contribute with 50% of the binding energy (Færgeman, et.al. 1996; Biochemistry, 35, 14118-26). ACBP does not bind fatty acids, nucleotide, prostaglandins and a number of other compound tested (Rosendal, et. al., 1993, Biochem J. 290,321-326). The high specificity makes the constructs very suitable for both *in vitro* and *in vivo* studies. The present work demonstrate the values of the constructs for *in vitro* determination of free acyl-CoA concentration. It is also envisaged and within the scope of the present invention to use the probes for *in vivo* studies.

The heterologous peptide preferably comprises a peptide having a high affinity for hydrophobic Coenzyme A esters, such as an acyl Coenzyme A binding protein or domain. Surprisingly, it has been found that the peptide conserves its high binding affinity for hydrophobic CoA esters even though the signal moiety is bound to the peptide. The peptide may even be bound to a carefully selected amino acid residue in the binding domain of the peptide, and still perform a high affinity binding to hydrophobic CoA esters.

The signal moiety preferably comprises an environmentally sensitive compound capable of emitting different signals in response to different environments. It is also surprising that the signal moiety retains its environmentally dependent signalling properties even though it is bound to a peptide according to the invention.

Through careful manipulation of the site for binding the signal moiety to the peptide, constructs may be modelled that are selective for one or for a group of species of hydrophobic CoA esters. Furthermore, through careful manipulation of the amino acid sequence in the peptide, especially in the binding domain of the peptide, constructs with a specific binding affinity for one species or for a group of species of hydrophobic CoA esters may be manufactured.

In a third aspect the invention relates to a kit for detection of the concentration of a hydrophobic Coenzyme A ester in a sample comprising at least a first construct according to the invention, and a sample compartment for application of the sample.

The kits may be laid out in different ways for different applications and provide an easy and convenient way for performing the method for determination according to the invention without requirement for expensive and sophisticated equipment such as equipment for gas chromatography and often without any need for pre-treatment. Thus it is expected that the kits will be useful for performing assays in clinics for diagnosis, on farms for diagnosis of animal husbandry and/or for quality control of milk, in factories for quality control of lipid or fatty acid containing materials and/or products, for analysis of food, feed, blood, urine, milk, or other physiological fluids.

In yet another aspect, the invention relates to a nucleotide sequence encoding the heterologous peptide comprised in the construct according to the invention, an expression vector and a cell comprising this nucleotide sequence.

The heterologous peptide making up one part of the construct may conveniently be produced using recombinant molecular techniques.

Definitions

Throughout the present application the term concentration is meant to include any concentration including 0. Thus it is an object of the present invention to measure the presence and or concentration of a given CoA ester or of CoA esters in a sample.

By specificity of a give construct with respect to a given CoA ester is meant specificity in the detected signal. This specificity may arise from a binding specificity but may also or additionally be caused by a signal specificity. Some constructs may thus bind a larger group of CoA esters but only produce a detectable signal in response to binding of one species or a group of CoA ester species. This is termed signal specificity.

The term ligand is used to designate a hydrophobic CoA ester capable of binding to a construct according to the invention. In a chemical sense the CoA ester may be regarded a ligand.

By "hydrophobic Co-enzyme A ester" is meant a Co-enzyme A ester, wherein the organic acid component of the acid is hydrophobic. In the sense of the present invention, the term also comprises CoASH as well as CoA esters of less hydrophobic carboxylic acids such as formic, acetic and butyric acid.

By free unbound CoA esters is meant the true free and unbound CoA esters. When the ester is first made from a free acid, it may not be unbound in a very strict sense, since it may be delivered to the binding construct directly from an acyl-Coenzyme A ligase. These CoA esters are also included in the term free unbound CoA esters for the purpose of the present invention.

By a signal is meant any signal detectable by detection techniques know to those skilled in the art. A signal – particularly a first signal within the meaning of the present invention – may also be 0.

Figures

Fig 1. Alignment of 30 ACBP sequences (SEQ ID NO 1 – 30). The alignments are done to the bovine sequence with residues Ser 1 to Ile 86. The lengths of the other sequences are indicated as a subscript after the last residue shown and the four helices of bovine ACBP are shown as boxes above the sequences. Conserved Class 1 residues are present in 18 out of the 21 l- and b-ACBPs and are highlighted by black boxes. Conserved class 2 residues are hydrophobic residues (either M/L/H/P/A/F/Y/V/I) in all l- and b-ACBP sequences and in at least 27 out of all 30 sequences and are highlighted by grey boxes. Cysteines are in white text in grey boxes. Yeast(1) is from Saccharomyces cervisiae and Yeast(2) from Saccharomyces monoasensis and from Saccharomyces pastoranis (identical).

Fig.2 shows a graphical description of measured fluorescence intensity in the 400 to 550 nm range resulting from titration with different levels of CoA, C4-CoA, C8-CoA C12-CoA, C16-CoA and C20-CoA with the badan derivative of M24C-bovine ACBP (Fluorescence Acyl-CoA Sensor 1 (FACS1)).

Fig.3 shows a graphical description of measured fluorescence intensity in the 400 to 550 nm range resulting from titration with different levels of CoA, C4-CoA, C8-CoA

C12-CoA, C16-CoA and C20-CoA with the badan derivative of A53C-bovine ACBP (Fluorescence Acyl-CoA Sensor 2 (FACS2)).

Fig. 4 shows isolelectrical point shift by bovine ACBP, M24C-bovineACBP and M24C-badan-bovine ACBP. Isoelectric focusing gels (PhastGel™ IEF 3-9) demonstrating the acyl-CoA binding profile of (a) r-bov ACBP, (b) A53C-badan and (c) M24C-badan. All the gels were prepared with ligands (1-7) in four-fold molar excess over protein. The band seen in lane 1 illustrates the unbound protein, which had a pI around 6 (confer with Broad pI Calibration Kit (pH 3-10)). The protein-ligand complex shifted to a pI around 3.8. Legend: 1: Water, 2: CoA-SH, 3: C4-CoA, 4: C8-CoA, 5: C12-CoA, 6: C16-CoA, 7: C20-CoA, 8: Broad pI Calibration Kit (pH 3-10).

Fig.5 show quantitative determination of the concentration of total fatty acid in blood serum using FACS1 in combination with acyl-CoA synthetase. For experimental details see the text. **A:** Measurement of palmitoyl-CoA formed from palmitic acid bound to bovine serum albumin. FACS1 (3μM) was incubated with the indicated amount of albumin bound Palmitic acid in the reaction mixture as described in the text. Excitation at 400 nm and emission reading at 470 nm. **B:** Measurement of total non-esterified free fatty acid in human serum. FACS1 (4 μM) was incubated with the indicated amounts of serum in the reaction mixture as described in the text. Excitation at 400 nm and emission reading at 470 nm.

Fig.6 Calculation of free acyl-CoA concentration in solutions of bovine ACBP titrated with different concentrations of palmitoyl-CoA in the presence of FACS1. For calculation details se the text.

Fig. 7 shows an overview of the different hydrophobic analytes that may be assayed according to the invention, together with appropriate pre-treatment steps.

Detailed description of the invention

The assay of this invention involves the single determination of signal intensity such as fluorescence intensity of signalling acyl CoA binding proteins (ACBP) such as fluorescent ACBP added to any aqueous solutions. The method directly determines the concentration of free acyl-CoA the activated form of fatty acids. If desired the

method can determine the total fatty acid concentration in any biological solution when linked to acyl-CoA synthetase (ACS).

The principles and exemplary methods for constructing probes as described and defined herein and methods for measuring acyl-CoA levels is described in details below. Using these principles three different fluorescent-ACBP derivatives have been constructed. This has been done using three site directed mutated bovine ACBP (Met24_24Cys, Ala53_53Cys and Fe49_49Cys) derivatised with badan (Molecular Probes). Two of these Met24_24Cys (FACS1) and A53_53Cys (FACS2) can serve as acylCoA probes. To date the FACS1 is a preferable probe for acyl-CoA esters with from 14- to 20-carbons in the acyl-chains, with highest sensitivity to C16-CoA and FACS2 a preferable probe for C8- to C12 CoA esters. The repertoire of possible variants of the biosensor includes mutations of all the amino acid residues lining the binding cavity which include Phe-49, Met24, Leu-25, Ala-53, Asp-21, Lys-50, Lys-54, Lys-18, pro-19, Ala-9, Tyr-31, Lys-32, Tyr-28, Tyr-73, Val-12, Lys-13, Leu-15; Ile-27.

Neither FACS1 or FACS2 respond significantly to binding of free CoA which makes both sensors suitable for measuring acyl-CoA synthetase activity. Occupying, partly, the binding site by derivation of the mutated amino acid residue with a fluorescent group would be expected to partly perturb the acyl-CoA binding therefore altering the acyl-CoA binding constant. Surprisingly the replacement of the $-CH_2-S-CH_3$ part Met-24 with the badan group only slightly affected binding affinity. The K_D C14-CoA binding is 16nM and 8 nM for native bovine ACBP and FACS1 respectively. However as long as the derivatised molecule can bind the acyl-CoA ester it can still function as an acyl-CoA probe. As long as its dynamic range is sufficient, the acyl-CoA and CoA levels, over a wide range, including those that are physiological, can be measured. This range can be further broadened by introducing additional mutations (Kragelund et al, 1999, Biochemistry 38 (8) pp 2386-94) or by in deleting or inserting one or more amino acid residues as seen in *Plasmodium falciparum* ACBP (unpublished data).

A costruct is prepared by any of the techniques describe below, or other techniques that can, using the guidance of this disclosure, be adapted to such a preparation. The construct comprises a heterologous peptide that has been labelled with a signal

moiety that, when so labelled specifically binds hydrophobic-CoA esters and exhibits one signal when unbound and a measurably different signal when bound to hydrophobic-CoA esters and the signal difference being detectable. Native acyl-CoA binding proteins (ACBP) or mutated ACBP can be used to provide CoA and hydrophobic-CoA ester reactive binding sites.

The heterologous peptide

Acyl Coenzyme A binding protein (ACBP)

The heterologous peptide comprised in the construct according to the invention preferably comprises an acyl-CoenzymeA binding protein, a variant or functional equivalent thereof. Acyl-Coenzyme A binding proteins (ACBPs) are known in the art from a wide variety of species including animals, plants and lower organisms. Wild-type ACBP is an 86-103 residue protein with a highly conserved amino acid sequence. It has been isolated from a wide range of species ranging from yeasts and plants to reptiles and man, but also several proteins translated from gene sequences, especially from Caenorhabditis elegans, have been suggested. A total of 30 sequences are disclosed in Figure 1.

From the alignment, at least four groups of ACBP can be identified. The first group is the generally expressed ACBP isoform, first isolated from bovine liver (l-ACBP, SEQ-ID NO 30). In their wild type form these ACBPs contain no cysteines and are 86-92 residues long. The second group is the testis specific isoform (t-ACBP) also called endozepine-like protein (ELP). T-ACBPs have now been isolated from three different species and these three all wild-type t-ACBPs contain three cysteines. A putative third group may be a brain specific isoform of ACBP (b-ACBP) which has been deduced from gene sequences from duck and frog brain and which contain in their wild type form one single cysteine at position 43. The fourth group of native ACBP is a group of longer sequences with up to 533 amino acids. Some of these longer sequences are suggested to be membrane bound isoforms (m-ACBP), whereas others remain to be isolated as proteins. Many of these longer forms comprise cysteine(s).

The construct according to the invention preferably comprises an acyl-Coenzyme A binding protein such as an acyl-CoA binding protein comprising an amino acid sequence from the sequences of Figure 1 (SEQ-ID NO 1-30) a variant or functional equivalent thereof.

Using the sequences (SEQ ID NO 1-30) the skilled protein chemist may easily identify homologous proteins in other species and even novel proteins having essentially the same affinity for CoA esters of hydrophobic acids. All these proteins and their functional variants are within the scope of the present invention.

The heterologous peptide of the construct may also preferably comprise an acyl-CoenzymeA binding domain. This domain could be isolated from a larger protein such as those shown in figure 1 (SEQ ID NO 1, 4, 5, 6, 7, 11) or from homologous proteins from those and other species.

According to a preferred embodiment of the invention the heterologous peptide comprises a modified form of bovine ACBP (SEQ ID NO 30), a variant or functional equivalent thereof. A number of constructs have been produced based on bovine ACBP and have shown to work well under laboratory conditions.

The linkage between the peptide and signal label

The signal moiety or signal label may be bound to the heterologous peptide via a cystein residue for binding the signal moiety. This cystein could be natively present in the construct or be introduced via substitution or addition.

Another possibility is that signal label is bound to a lysine residue, which likewise may be present in a native peptide or introduced by substitution or addition of an amino acid residue.

Methods are well known in the art for binding compounds having specific groups to the side chains of cystein and lysin residues. However, it also lies within the scope of the present invention to link the signal moiety to the side chain of any other amino acid residue in the presence of a suitable and specific reaction. Such reaction may comprise but is not limited to nucleophilic substitution or addition, or electrophilic

substitution or addition reaction, esterification, thioesterification, condensation reactions, amide reactions. Preferably the reaction is a specific reaction, so that the number and the position of signal moieties linked to the peptide is closely controlled. Such other amino acid residues include but are not limited to trp, ser, thr, tyr, asp, glu, his. Preferably, the linkage should be performed without substantially altering the signalling properties of the signal moiety.

Preferably the heterologous peptide comprises only one residue of the type to which the signal moiety is to be linked. In the presence of two or more residues of the same type such as two or more cysteines, a signal moiety may be bound to both of the cystein residues.

If more than one signal moiety is to be linked to the construct and if these more than one signal moieties are different, they may advantageously be linked to different amino acid residues in order to facilitate the specificity of the linkage.

The amino acid residue, to which the signal label is bound may be selected from the amino acid residues aligning the acyl Coenzyme A binding domain. The residue may also be selected from the amino acid residues having van der Waals' contact with a bound hydrophobic Coenzyme A ester or it may be selected from the amino acid residues being within 5 Å from a bound hydrophobic Coenzyme A ester.

The residue may likewise be selected from the amino acid residues making up the α-helices of the heterologous peptide.

More specifically the heterologous peptide may comprise the bovine ACBP (SEQ ID NO 30) and the native amino acid being replaced by a cystein residue is preferably selected from the group consisting of Phe-49, Met24, Leu-25, Ala-53, Asp-21, Lys-50, Lys-54, Lys-18, pro-19, Ala-9, Tyr-31, Lys-32, Tyr-28, Tyr-73, Val-12, Lys-13, Leu-15, Ile-27. More preferably the amino acid being substituted by a cystein residue is selected from the group consisting of Met-24 and Ala-53.

The position of the amino acid residue carrying the signal label may determine the specificity of the construct with respect to the hydrophobic CoA esters. Through careful selection of the residue carrying the signal label, constructs being specific for

a specific hydrophobic CoA ester or being specific for a group of hydrophobic CoA esters may be designed.

However, other changes, such as substitution, deletion or addition, to the amino acid sequence of the heterologous peptide may also affect the binding properties of the peptide in the sense that two constructs having the signal moiety bound to the same amino acid residue but differing at another position, may have different binding affinity towards a hydrophobic CoA ester. Similarly two such different constructs may bind the same CoA esters but exhibit different signals in response to binding different CoA esters.

Variants

The amino acid sequence of the heterologous peptide preferably has at least 30% sequence identity to one of the sequences (SEQ ID NO 1 – 30) of Figure 1, such as at least 40 % sequence identity, for example at least 50 % sequence identity, such as at least 55% sequence identify, for example at least 60% sequence identity, such as at least 65 % sequence identity, for example at least 70 % sequence identity, such as at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, such as at least 91 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity.

A variant of the sequences of Figure 1 (SEQ ID NO 1-30) according to the invention may appropriately be defined with reference to the four α-helices, which the variants preferably comprise. These are in the following termed A1, A2, A3 and A4. These four helices are preferably linked together by an A1-A2 linking peptide, an A2-A3 linking peptide, and an A3-A4 linking peptide. The variants preferably also comprise a N-terminal peptide and a C-terminal peptide.

Preferred variants are in the following described with reference to these 8 constituents.

A1 preferably comprises 12 amino acids capable of forming an α-helix, which may be described by the general formula: X-1-X-X-X-2-X-X-3-X-X-4, where X denotes any individually selected amino acid; 1 preferably denotes a leu but may also denote ala; 2 preferably denotes ala but also may denote cys, gln, val, or lys; 3 preferably denotes val but also may denote ala, ile, leu, or ser; and 4 preferably denotes leu.

A2 preferably comprises 16 amino acids capable of forming an α-helix, which may be described by the general formula: X-X-X-1-X-2-3-X-X-4-5-6-7-8-9-10, where X denotes any individually selected amino acid. 1 preferably denotes leu, but also may denote K or M; 2 preferably denotes a hydrophobic residue, more preferably an ile residue, but it may also denote a val, a leu, a phe or met residue; 3 preferably denotes a tyr residue; 4 preferably denotes a hydrophobic residue, more preferably a tyr or phe residue; 5 preferably denotes a lys residue; 6 preferably denotes a gln residue but also may denote an ile residue; 7 preferably denotes a ala residue, but also may denote a gly or ser residue; 8 preferably denotes a thr residue, but also may denote a ser or lys residue; 9 preferably denotes a val residue but also may denote an ala, phe, gln, ala, ile, ser or glu residue; 10 preferably denotes a gly residue.

A3 preferably comprises 12 amino acids capable of forming an α-helix, which may be described by the general formula: X-1-2-3-X-4-5-X-X-X-X-6, where X denotes any individually selected amino acid; 1 preferably denotes a hydrophobic amino acid residue more preferably an ala residue, but it may also denote a tyr, a lys or a met residue; 2 preferably denotes a lys residue; 3 preferably denotes a trp residue, but it may also denote a phe or a tyr residue; 4 preferably denotes an ala residue but may also denote a ser residue; 5 preferably denotes a trp residue; and 6 preferably denotes a gly residue, but may also denote an asn, a ser, an asp, or an ala residue.

A4 preferably comprises 20 amino acids capable of forming an α-helix, which may be described by the general formula: X-1-X-2-X-X-X-3-4-X-X-5-X-X-6-X-X-X-X-X, where X denotes any individually selected amino acid; 1 preferably denotes a glu residue but may also denote an asp or a met; 2 preferably denotes an ala residue; 3 preferably denotes a tyr residue; 4 preferably denotes a hydrophobic residue, more preferably an ile, a val, or an ala residue; 5 preferably denotes a val residue, but

may also denote an ala, a leu, a met or an ile residue; 6 preferably denotes a leu residue, but may also denote a met or an ile residue.

The A1-A2 linking peptide preferably comprises from 6 to 10 amino acid residues. When the A1-A2 linking peptide consists of 6 amino acids, amino acid residue number 3 or 4 preferably is a pro residue. When it consists of 10 amino acid residues, amino acid number 5 or 8 preferably is a pro residue.

The A2-A3 linking peptide preferably comprises 14 to 15 amino acid residues capable of forming an overhand loop which may be described by the general formula: X-1-X-2-X-X-X-3-4-5-6-X-7-X-X, wherein X denotes any individually selected amino acid residue. 1 may denote a cystein residue. 2 preferably denotes no amino acid resulting in a peptide of 14 residues, however when present it preferably denotes a pro residue. 3 preferably denotes a pro residue. 4 preferably denotes a gyl residue, but it may also denote a pro residue, a tyr residue or a ser residue. 5 preferably denotes a hydrophobic residue, more preferably a met residue, a leu residue, a phe residue, an ile residue or an ala residue. 6 preferably denotes a hydrophobic residue, more preferably a leu residue, a phe residue, a met residue or a trp residue. However 6 may also denote a thr residue. 7 preferably denotes a hydrophobic residue, more preferably a phe residue, a leu residue, a met residue, a pro residue, a val residue or an ile residue.

The A3-A4 linking peptide preferably comprises 2 amino acids, having the general formula X-1, wherein X denotes any individually selected amino acid, and 1 preferably denotes a ser residue, but it may also denote an ala residue, a thr residue, an asp residue, or a pro residue.

Variants of the sequences in figure 1 may also comprise a C-terminal peptide and/or a N-terminal peptide. Full length proteins corresponding to sequences in Figure 1 thus further comprise N terminal peptides of 3, 24 and 41 amino acids, and C terminal peptides of 19, 33, 52, 117, 276, 327, and 403 amino acids. Thus it is conceivable to the skilled person that the length of the peptide may much longer than the length of the acyl-CoA binding domain displayed in Figure 1 without substantially altering the binding capability of the peptide. A specific type of peptide that may be added to a terminal, preferably to the N-terminal end of a peptide

according to the invention is an affinity tag, such as a His tag. Experiments have shown that it is possible to add a poly His tag comprising e.g. 6 His residues and a linker residue without substantially altering the binding capabilities of the peptide. It is thus not necessary to cleave off the poly His tail after purification.

The peptide may furthermore comprise a proteinase cleavage site for cleaving off a tag, which is only used during purification of the peptide.

It is expected that by making substitutions, deletions and/or insertions of amino acid residues, the specificity of the heterologous peptide with respect to CoA esters is changed and/or the signal emitted or detected is changed.

Accordingly, a variant of the sequences in Figure 1 or fragments thereof according to the invention may comprise, within the same variant of the sequences in Figure 1 or fragments thereof or among different variant of the sequences in Figure 1 or fragments thereof, at least one substitution, such as a plurality of substitutions introduced independently of one another. Variants of the sequences in Figure 1 or fragments thereof may thus comprise conservative substitutions independently of one another, wherein at least one glycine (Gly) of said variants of the sequences in Figure 1 or fragments thereof of the sequences in Figure 1 is substituted with an amino acid selected from the group of amino acids consisting of Ala, Val, Leu, and Ile, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one of said alanines (Ala) of said variant of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Val, Leu, and Ile, and independently thereof, variant of the sequences in Figure 1 or fragments thereof, wherein at least one valine (Val) of said variant of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Leu, and Ile, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one of said leucines (Leu) of said variant of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Val, and Ile, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one isoleucine (Ile) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the

group of amino acids consisting of Gly, Ala, Val and Leu, and independently thereof, variants of the sequences in Figure 1 or fragments thereof wherein at least one of said aspartic acids (Asp) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Glu, Asn, and Gln, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one of said phenylalanines (Phe) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Tyr, Trp, His, Pro, and preferably selected from the group of amino acids consisting of Tyr and Trp, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one of said tyrosines (Tyr) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Phe, Trp, His, Pro, preferably an amino acid selected from the group of amino acids consisting of Phe and Trp, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one of said arginines (Arg) of said fragment of the sequences in Figure 1 is substituted with an amino acid selected from the group of amino acids consisting of Lys and His, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one lysine (Lys) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Arg and His, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one of said aspargines (Asn) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Gln, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one glutamine (Gln) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Asn, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one proline (Pro) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Phe, Tyr, Trp, and His, and independently thereof, variants of the sequences in Figure 1 or fragments thereof, wherein at least one of said cysteines (Cys) of said variants of the sequences in Figure 1 or fragments thereof is substituted with an amino acid

selected from the group of amino acids consisting of Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, and Tyr.

It is clear from the above outline that the same variant or fragment thereof may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

The addition or deletion of an amino acid may be an addition or deletion of from 2 to 10 amino acids, such as from 10 to 20 amino acids, for example from 20 to 30 amino acids, such as from 40 to 50 amino acids. However, additions or deletions of more than 50 amino acids, such as additions from 10 to 100 amino acids, addition of 100 to 150 amino acids, addition of 150-250 amino acids, are also comprised within the present invention.

It will thus be understood that the invention concerns a heterologous peptide comprising at least one fragment of the sequences in Figure 1 capable of binding at least one species of hydrophobic CoA esters, including any variants and functional equivalents of such at least one fragment.

The heterologous peptide according to the present invention, including any functional equivalents and fragments thereof, may in one embodiment comprise less than 250 amino acid residues, such as less than 240 amino acid residues, for example less than 225 amino acid residues, such as less than 200 amino acid residues, for example less than 180 amino acid residues, such as less than 160 amino acid residues, for example less than 150 amino acid residues, such as less than 140 amino acid residues, for example less than 130 amino acid residues, such as less than 120 amino acid residues, for example less than 110 amino acid residues, such as less than 100 amino acid residues, for example less than 90 amino acid residues, such as less than 85 amino acid residues, for example less than 80 amino acid residues, such as less than 75 amino acid residues, for example less than 70 amino acid residues, such as less than 65 amino acid residues, for example less than 60 amino acid residues, such as less than 55 amino acid residues, for example less than 50 amino acid residues.

Fragments

A fragment comprising the acyl-CoA binding region of the native sequences in Figure 1 is particularly preferred. However, the invention is not limited to fragments comprising the acyl-CoA binding region. Deletions of such fragments generating functionally equivalent fragments of the sequences in Figure 1 comprising less than the acyl-CoA binding domain are also comprised in the present invention. Functional equivalents of the sequences in Figure 1 peptides, and fragments thereof according to the present invention, may comprise less or more amino acid residues than the acyl-CoA binding region.

"Functional equivalency" as used in the present invention is according to one preferred embodiment established by means of reference to the corresponding functionality of a predetermined fragment of the sequences in Figure 1. More specifically, functional equivalency is to be understood as the ability of the functional equivalent to bind specifically to CoA esters of hydrophobic acids or to at least one species of CoA esters of hydrophobic acids. By specific binding is meant that the K_D of the complex between the CoA ester and the heterologous peptide is below 2 µM, such as below 1.5 µM, for example below 1.0 µM, preferably below 500 nM, more preferably below 200 nM such as below 100 nM, for example below 90 nM, such as below 80 nM, for example below 70 nM, such as below 60 nM, for example below 50 nM, such as below 40 nM, for example below 30 nM, such as below 20 nM, for example below 15 nM, such as below 10 nM, for example below 8 nM, such as below 7 nM, for example below 6 nM, such as below 5 nM, for example below 4 nM, such as below 3 nM, for example below 2 nM, such as below 1 nM.

Functional equivalents of variants of the sequences in Figure 1 will be understood to exhibit amino acid sequences gradually differing from the preferred predetermined sequence, as the number and scope of insertions, deletions and substitutions including conservative substitutions increases. This difference is measured as a reduction in homology between the preferred predetermined sequence and the fragment or functional equivalent.

All fragments or functional equivalents of ACBPs are included within the scope of this invention, regardless of the degree of homology that they show to a preferred predetermined sequence of ACBP. The reason for this is that some regions of the

sequences in Figure 1 are most likely readily mutatable, or capable of being completely deleted, without any significant effect on the binding activity of the resulting fragment.

A functional variant obtained by substitution may well exhibit some form or degree of native activity of the sequences in Figure 1, and yet be less homologous. If residues containing functionally similar amino acid side chains are substituted. Functionally similar in this respect refers to dominant characteristics of the side chains such as hydrophobic, basic, neutral or acidic, or the presence or absence of steric bulk. Accordingly, in one embodiment of the invention, the degree of identity between i) a given the sequences in Figure 1 fragment capable of effect and ii) a preferred predetermined fragment, is not a principal measure of the fragment as a variant or functional equivalent of a preferred predetermined the sequences in Figure 1 fragment according to the present invention.

The homology between amino acid sequences may be calculated using well known algorithms such as BLOSUM 30, BLOSUM 40, BLOSUM 45, BLOSUM 50, BLOSUM 55, BLOSUM 60, BLOSUM 62, BLOSUM 65, BLOSUM 70, BLOSUM 75, BLOSUM 80, BLOSUM 85, or BLOSUM 90.

Fragments sharing at least some homology with the sequences in Figure 1 fragment are to be considered as falling within the scope of the present invention when they are at least about 40 percent homologous with the ACBP or fragment thereof, such as at least about 50 percent homologous, for example at least about 60 percent homologous, such as at least about 70 percent homologous, for example at least about 75 percent homologous, such as at least about 80 percent homologous, for example at least about 85 percent homologous, such as at least about 90 percent homologous, for example at least 92 percent homologous, such as at least 94 percent homologous, for example at least 95 percent homologous, such as at least 96 percent homologous, for example at least 97 percent homologous, such as at least 98 percent homologous, for example at least 99 percent homologous with the sequences in Figure 1 or fragments thereof. According to one embodiment of the invention the homology percentages refer to identity percentages.

Additional factors that may be taken into consideration when determining functional equivalence according to the meaning used herein are i) the ability of antisera raised against the peptides of Figure 1 to detect a fragment of the sequences in Figure 1 according to the present invention, or ii) the ability of the functionally equivalent fragment to compete with the sequences in Figure 1 in a CoA ester binding assay.

Conservative substitutions may be introduced in any position of a preferred predetermined ACBP peptide or fragment thereof. It may however also be desirable to introduce non-conservative substitutions, particularly, but not limited to, a non-conservative substitution in any one or more positions.

A non-conservative substitution leading to the formation of a functionally equivalent fragment of the sequences in Figure 1 would for example i) differ substantially in polarity, for example a residue with a non-polar side chain (Ala, Leu, Pro, Trp, Val, Ile, Leu, Phe or Met) substituted for a residue with a polar side chain such as Gly, Ser, Thr, Cys, Tyr, Asn, or Gln or a charged amino acid such as Asp, Glu, Arg, or Lys, or substituting a charged or a polar residue for a non-polar one; and/or ii) differ substantially in its effect on polypeptide backbone orientation such as substitution of or for Pro or Gly by another residue; and/or iii) differ substantially in electric charge, for example substitution of a negatively charged residue such as Glu or Asp for a positively charged residue such as Lys, His or Arg (and vice versa); and/or iv) differ substantially in steric bulk, for example substitution of a bulky residue such as His, Trp, Phe or Tyr for one having a minor side chain, e.g. Ala, Gly or Ser (and vice versa).

Substitution of amino acids may in one embodiment be made based upon their hydrophobicity and hydrophilicity values and the relative similarity of the amino acid side-chain substituents, including charge, size, and the like. Exemplary amino acid substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition to the variants described herein, sterically similar variants may be formulated to mimic the key portions of the variant structure and that such

compounds may also be used in the same manner as the variants of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

Signal labels

The label, which is linked to the heterologous peptide according to the invention, may be termed a signal moiety or a signal label. In the following these terms are used interchangably.

The signal label is preferably linked to the heterologous peptide via a covalent linkage. Such a linkage could be made e.g. between the label and a cystein or a lysin residue in the peptide. The signal label is preferably of the type that changes its signal in response to a change in the environment and/or conformation, e.g. a change in the polarity of the environment. The signal label may thus comprise a fluorescent label, a chromogenic label, a chemoluminescent label, or a photoluminescent label.

Exemplary fluorescent labels are described below. The nature of the fluorescent label is not critical however, it need only to be capable of being attached to the specific heterologous peptide and, when attached emit fluorescence measurably different when the protein is bound with a CoA ester compared to the fluorescence emitted when unbound. The mode of detection is also not critical. In other words, the label and the mode of detection are not critical limiting factors in this invention.

The fluorescent moiety preferably comprises a compound selected from the group consisting of acrylodan; 5-dimethylaminonaphtalene-1-sulfonyl aziridine (danzyl aziridine); 4-[N-[2-iodoacetoxy)ethyl]-N-methylamino]-7-nitrobenz-2-oxa 1,3 diazole ester (IANBDE); 4-[N-[2-iodoacetoxy)ethyl]-N-methylamino]-7-nitrobenz-2-oxa 1,3 diazole amide (IANBDA); 6-acryloyl-2-dimtheylaminonaphtalene (acrylodan); N-(7-chlorobenz-2-oxa-1,3-diazyl-4-yl)sulfonyl morpholine; 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride); didansyl-L-cystine; N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide); 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F); 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole

(NBD fluoride); 2-(4'-(iodoacetamido)anilino)naphtalene-6-sulfonic acid, sodium salt (IAANS); 5-(((2-iodoacetyl)amino)ethyl)amino)naphtalene-1-sulfonic acid (1,5-IAEDANS); 2-(4'-maleimidylanilino)naphtalene-6-sulfonic acid (MIANS); N-(1-pyreneethyl)iodoacetamide; N-(1-pyrene)iodoacetamide; N-(1-pyrene)maleimide; N-(1-pyrenemethyl)iodoacetamide (PMIA amide); 1-pyrenemethyl iodoacetate (PMIA ester); N-(1-pyrenepropyl)iodoacetamide); 1-(2,3-epoxypropyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium trifluoromethanesulfonate (PyMPO epoxide); erythrosin-5-iodoacetamide; fluorescein-5-maleimide; 5-Iodoacetamidofluorescein (5-IAF); 6-iodoacetamidofluorescein (6-IAF); 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate (PyMPO maleimide); Oregon Green™ 488 iodoacetamide "mixed isomers"; tetramethylrhodamine-5-iodoacetamide (5-TMRIA) "single isomer"; tetramethylrhodamine-5-maleimide "single isomer"; tetramethylrhodamine-6-maleimide "single isomer"; Texas Red® C_5 bromoacetamide; Texas Red® C_2 maleimide. More preferably the fluorescent moiety comprises Badan.

The fluorescent moiety may also comprise derivatives of the compounds mentioned above.

Furthermore, the construct may comprise a linker molecule for linking the fluorescent moiety with the peptide. The role of the linker molecule may be to facilitate the chemical bonding of the signal moiety to an amino acid residue in the peptide or the role may be to position the spacer moiety in relation to the peptide.

The construct may also further comprise a second signal moiety. The second signal moiety may similarly comprise a fluorescent label, a chromogenic label, a chemoluminescent label, or a photoluminescent label. Preferably the second signal moiety comprises a compound selected from the group of fluorescent labels listed above. The first and second signal moiety may comprise the same compound or they may preferably comprise two different compounds.

The effect of binding a second signal label to the heterologous peptide may be to change the specificity of the construct vis a vis the ligand and/or to affect the signal change upon binding of the ligand. By having e.g. two flourescent labels attached to a heterologous peptide according to the invention, it may be possible not only to

obtain an increase in the emission at one wavelength but in addition a simultaneous decrease in the emission at another wavelength compared to unbound construct. Thereby a more precise signal can be recorded. The inventors also envisage that binding of different ligands to a construct comprising two or more signal labels will result in differential change in the emission at two different wavelengths, thereby allowing identification of the ligand bound to the construct through a mathematical combination of emission change at two or more different wavelengths. The second ligand is preferably bound to an amino acid positioned, so that the ligand is moved from a hydrophobic to a hydrophilic environment upon binding of the hydrophobic CoA ester.

The difference in fluorescence between a solution comprising a construct according to the invention and the solution comprising a construct-hydrophobic CoA ester complex is detected or measured. The change in fluorescence is related to the amount of free hydrophobic-CoA esters in the solution. This may be a qualitative relationship i.e., hydrophobic-CoA ester present or not present above some threshold level, but in most instances the fluorescence change is related quantitatively to the concentration of hydrophobic CoA ester. Once the hydrophobic-CoA ester dissociation constant (K_D) has been determined, the concentration of the hydrophobic CoA ester can be calculated from the detected signal.

The signal

The detected signal according to the invention may comprise a fluorescence signal, a chromogenic signal, a chemiluminiscense signal, or a photoluminiscense signal.

The detected signal may comprise the second signal (which is the signal detectable after binding of the CoA ester to the construct). In this case the first signal preferably is essentially zero so that the difference between the signals do not have to be calculated. Alternatively, the detected signal may comprise the difference between the first and the second signal or the detected signal may comprise a mathematical combination between two different signals such as between two signals emitted or detected at two different wavelengths.

Preferably, the detected signal is essentially proportional to the amount of hydrophobic Coenzyme A ester in the sample such as being essentially proportional to the amount of at least one species of Coenzyme A ester in the sample.

According to an especially preferred embodiment of the invention, the at least one species of Coenzyme A ester for which the detected signal is essentially proportional to its amount comprises a species selected from the group consisting of Coenzyme A esters with a C2 acyl group, a C4 acyl group, a C6 acyl group, a C8 acyl group a C10 acyl group, a C12 acyl group, a C14 acyl group, a C16 acyl group, a C18 acyl group, a C20 acyl group, a C22 acyl group, a C24 acyl group, a C26 acyl group, a saturated acyl group, a mono-unsaturated acyl group, a polyunsaturated acyl group, an acyl group comprising a cis double bond, an acyl group comprising a trans double bond, an acyl group comprising a ring structure, an acyl group comprising a side chain.

Thereby it is possible to selectively detect the amount of one species or a group of species, which are related in terms of similar length or similar configurations in the side chain.

Similarly, the detected signal from a first species of hydrophobic Coenzyme A ester may be essentially 0 (i.e. no binding to the construct) and the detected signal from a second species of hydrophobic Coenzyme A may be essentially proportional to the amount of said second species in the sample (binding to the construct and thus signal).

The first species may comprise a saturated species and the second species may comprise an unsaturated species or vice versa. The first species may comprises a mono-unsaturated species and the second species a poly-unsaturated species or vice versa. The first species may comprise a species with a cis-double bond and the second species a trans-double bond or vice versa. The first species may comprise a double bond and the second species comprises a double bond in another position.

According to another embodiment of the invention the detected signal may be essentially proportional to the amount of a group of hydrophobic Coenzyme A esters in the sample. This group may comprise Coenzyme A esters with a C2-C6 acyl

group, Coenzyme A esters with a C8-C12 acyl group, Coenzyme A esters with a C12-C16 acyl group, Coenzyme A esters with a C16-C20 acyl group, Coenzyme A esters with a C12-C20 acyl group, Coenzyme A esters with a C22-C24 group, Coenzyme A esters with a C6-C10 acyl group, or a C10-C14 acyl group, or a C14-C18 acyl gruop, or a C18-C22 acyl group, or a C4-C8 acyl group, or a C8-C16 acyl group, or a C4-C12 acyl group, or an acyl group comprising more than 20 carbon atoms. Thus it is envisaged that it is possible to design a construct according to the invention which is specific for any one group of CoA esters having some chemical property in common. Through use of several constructs having specificity for different groups of species, differential analysis of a complex sample may be performed.

By careful design of two or more constructs each being specific for a different group of CoA species and each providing a measurably different signal when bound it may be possible to detect in one step the concentration of more than one group of species of CoA in a single sample. In order to make full use of this option, the two or more constructs should have a specific binding affinity of the different groups of CoA species and they should also measurably different signals upon binding of the CoA species.

Dissociation constant

As stated above, the dissociation constant, K_D, of the complex between C14-CoA and native ACBP is 16 nM, which indicates a very strong binding between the protein and the ligand. The K_D between hydrophobic CoA esters and the constructs according to the invention preferably is below below 2 μM, such as below 1.5 μM, such as below 1.0 μM preferably below 500 nM, more preferably below 200 nM, such as below 100 nM, for example below 90 nM, such as below 80 nM, for example below 70 nM, such as below 60 nM, for example below 50 nM, such as below 40 nM, for example below 30 nM, such as below 20 nM, for example below 15 nM, such as below 10 nM, for example below 8 nM, such as below 7 nM, for example below 6 nM, such as below 5 nM, for example below 4 nM, such as below 3 nM, for example below 2 nM, such as below 1 nM.

The K_D of the construct according to the invention may be determined with reference to one species of CoA ester, to a group of CoA esters or to CoA esters in general. In order to be able to detect specific species or groups of species of CoA esters the K_D with respect to this one species or group of species preferably is lower than the K_D of the same construct with respect to other CoA esters. By lower is preferably meant at least 10 times lower, more preferably at least 100 times lower.

Specificity of signal

Reference is made to Fig 2. and Fig 3. which depict the emission spectra of FACS1 and FACS2 titrated with increasing concentrations of CoA, C4-, C8-, C12-, C16- and C20-CoA esters. The addition of ligand and measurement of emission of FACS1 and FACS2 was performed as described in example 2. The increased addition of ligand to the mutated and modified proteins caused a proportional spectral change. The normal physiological binding profile was confirmed by direct binding studies using isoelectrical focusing as predicted in Fig 4. This demonstrates that mutation and fluorescent modification does not abolish the acyl-CoA binding characteristics of bovine ACBP. In fact FACS1 binds C14-CoA with essentially similar affinity (K_D = 16nM) as native bovine ACBP (K_D 8nM). The results clearly indicate that fluorescence emission at 470 nm may provide a measure for the concentration of free unbound long-chain(> C12)-acyl-CoA ester. The emission profile of FACS2 differed from that of FACS1 in that emission maximum was observed at 487 nm instead of 465 nm and that FACS2 exhibits highest sensitivity for C8- to C12-acyl-CoA and the probe hardly responded to CoA and C20-CoA binding. The lack of fluorescence response to C16-CoA and C20-CoA was not due to lack of binding, both acyl-CoA were shown to bind to FACS2 by isoelectrical focusing (Fig 4) and C16-CoA binding was confirmed by isothermal titration calorimetry.

These results clearly demonstrate that acyl-CoA sensor probes can be designed by engineering of the binding site at different locations with flourescent groups sensitive to differences in the environment. The two sensors presented herein together act as high sensitivity sensors in the chain length range from C8-CoA to C20-CoA. The Phe-49_Cys49 badan derivative of bovine ACBP which has the badan group exposed to the environment did not respond to addition of any of the ligands showing that the flourescent group preferably is located in the binding site in order to

respond to ligand binding. However, it is envisaged that the signal moiety may be located in any position, where a change in the hydrophobicity of the environment takes place upon binding of the CoA ester. The lack of or very low response of FACS2 and FACS1 respectively to CoA binding makes these sensors preferred high sensitivity sensor for any acyl-CoA producing enzyme including acyl-CoA synthetases. This makes FACS1 a very potent sensor in determination of total free fatty (FFA) acid concentration in any biological fluid following the conversion of these to acyl-CoA esters.

Pre-treatment of hydrophobic analytes other than hydrophobic CoA esters

The method, construct and kit according to the invention may be used for measuring the concentration of a number of different hydrophobic analytes. The analytes all have in common that it is possible to convert them via known and simple methods to hyrdophobic CoA esters, which are the keypoint linking these analytes together.

Reference is made to Figure 7, in which the various groups of possible analytes are described together with suitable steps to perform before measurement of the amount of hydrophobic CoA ester. In the figure, ellipsoids contain the name of the different groups of hydrophobic analytes, triacylglycerides, phospholipids, cholesterolesters, free fatty acids and acyl CoA esters. Arrows show the direction of the steps necessary for converting the analytes into hydrophobic acyl CoA esters. The conversion steps may be performed in different ways, but for illustrative purposes the name of a preferred enzyme capable of catalysing the conversion steps have been added in rectangles.

A key reaction in the analysis of all hydrophobic analytes is the conversion of free fatty acids to acyl CoA esters. Methods based on initial conversion of the FFA to acyl-CoA are well known in the art. However quantification of the synthesised acyl-CoA in all the reported methods rely on time and resource consuming enzyme linked assays.

In order to be able to measure the concentration and/or presence of free fatty acids and/or lipids and/or phospholipids, these compounds must first be converted into CoA esters. Therefore the assay may further comprise a step prior to binding of the

CoA esters with the construct, wherein hydrophobic acids in the sample are converted to hydrophobic Coenzyme A esters.

This conversion may conveniently be performed using enzymes such as acyl Coenzyme A ligase.

In all known FFA assays based on conversion of FFA to CoA esters pyrophosphatase is added to the sample together with acyl-CoA ligase and free CoA in order to drive the reaction in the direction of formation of CoA esters. By linking the cleavage of pyrophosphate liberated from CoA upon esterification to the acid group of the hydrophobic acid, to the esterification reaction, the overall reaction is rendered endothermic and essentially all hydrophobic acid is converted to hydrophobic CoA esters. However, because of the high binding affinity of the product of the esterification reaction towards the construct according to the present invention, this binding alone suffices to drive the esterification reaction. In the presence of the probe, the addition of pyrophosphatase may thus be dispensed with. In all other known assays, which include esterification of hydrophobic organic acids with CoA, pyrophosphatase is required to drive the reaction.

If fatty acids comprised in lipids are to be measured an additional step may be included during which triacylglycerides in the sample are converted to glycerol and free fatty acids. This hydrolysis is followed by esterification through acyl-CoA ligase.

The hydrolysis preferably is catalysed by lipase but it may also comprise acid or basic ester hydrolysis. Hydrolysis catalysed by lipase is by far the most gentle method and due to the specificity of the reaction the risk of uncontrolled and undesirable side reactions can be minimised. Thus lipase and the necessary reagents and co-factors may be added to the sample together with the components for the CoA binding assay.

In the case of phospholipids, the method preferably further comprises a prior step wherein phospholipids in the sample are converted to glycerol and free fatty acids. This is preferably performed using phospholipase A1 and/or phospholipase A2 but may likewise comprise acid or basic ester hydrolysis.

The inventors also envisage that the method may be used for estimation of the concentration of cholesterol esters in a sample such as a blood sample. The amount and type of cholesterol esters in blood is indicative of several diseases such as aterosclerosis and genetic defects such as familiar hypercholesterolemia. After cleaving cholesterolesters with an enzyme specific for cholesterolesters, the liberated free fatty acids may be combined with CoASH to form a CoA species that may be measured according to the present method.

Through combination of the various different pre-treatment steps, information concerning the type and amount of free fatty acids, CoA fatty acid esters, fatty acids making part of triacylglycerides and fatty acids constituting part of phospholipids in one and the same sample may be obtained.

Such combined assay may first comprise measurement of the amount of CoA esters in the sample using the construct according to the invention. By addition of acyl-CoA ligase the amount of free fatty acids may then be measured. Then the amount of triacylglyceride fatty acid may be measured by addition of lipase, and finally the amount of phospholipid fatty acids may be measured through addition of phospholipase A1 and/or phospholipase A2.

Applications

The sensitivity and the simplicity of the constructs according to the invention make them useful in a variety of applications. At present no other methods exist for determining free acyl-CoA concentration. The probes are able to monitor the rate of C8- to C20-acyl-CoA production by any such acyl-CoA producing reaction. The probes are also in combination with acyl-CoA synthetase able to monitor the release of fatty acids (C8 to C20) from fatty acid producing reactions. The advantage of the present probes for determining FFA in combination with acyl-CoA synthetase over the ADIFAB probe produced by Molecular Probes is that the FACS1 and FACS2 are specific for long (>C12) and the medium chain acyl-CoA (C8 to C12) respectively. Furthermore the method does not require knowledge about the concentration of fatty acid binding proteins such as albumin in the reaction mixture.

As illustrated above it will not be difficult for a skilled protein chemist following procedures presented herein to construct new CoA or acyl-CoA probes using the above and other variants of ACBP. It only requires introduction of a environmentally sensitive signalling group in a position in the binding site which undergoes environmentally changes upon ligand binding. In the present study the amino acid residues selected to be mutated and derivatised have been shown to interact directly with the acyl-chain of the bound ligand (Kragelund,et. al., 1999; Biochim Biophys Acta. 1441, 150-161). These residues are exposed to the solvent in the unbound protein. The down shift in emission spectra therefore represents a hydrophobic shift in the local environment upon ligand binding. The ligand binding site is an open bowl like cavity from which water is displaced and the hydrophobic binding pocket for the acyl-chain is formed by the protein and the CoA head group together upon ligand binding (Faergeman, et. al.,1996; Biochemistry. 35:14118-14126 ; Kragelund, et. al., 1193; J Mol Biol. 230,1260-1277). A more sensitive probe would be one where the environment of the flourescent group is undergoing more dramatic changes upon ligand binding.

The use of the probes presented herein is the only existing way to measure free acyl-CoA concentrations of the physiological important, highly amphiphatic, medium and long chain acyl-CoA esters. Long-chain acyl-CoA esters partition in to membranes, stick to proteins and test tube cell walls. All previous published methods measure total acyl-CoA concentration including the very small fraction of free acyl-CoA, the biological active fraction, which can only be measured with the probes invented herein. From the literature it is clear that knowledge of the free acyl-CoA concentration *in vivo* and *in vitro* conditions is the key to understand the function of these very important molecules in regulation of key cell functions including gene expression (Faergeman and Knudsen, 1997; Biochem J. 323 , 1-12). The advantage with the present probes are their high degree of specificity for hydrophobic-CoA esters only. The CoA head group determines the binding specificity of ACBP by interacting with specific amino acid residues in the binding site and contribute with 50% of the binding energy (Færgeman, et.al. 1996; Biochemistry, 35, 14118-26). ACBP does not bind fatty acids, nucleotide, prostaglandins and a number of other compound tested (Rosendal, et. al., 1993, Biochem J. 290,321-326). The high specificity makes the probes very suitable for both *in vitro* and *in vivo* studies. The present work demonstrate the values of the

FACS probes for *in vitro* determination of free acyl-CoA concentration. It is also envisaged and within the scope of the present invention to use the probes for *in vivo* studies.

The exemplary method, fluorescence ACBP, will also have wide applicability in studies of intracellular acyl-CoA transport, the role of acyl-CoA esters in fatty acid induced diseases and in enzymatic assays measuring total fatty acid concentration and the rate of fatty acid release from lipases, cells and lipid degradation in feed and food preparations.

The sample

The method and the assay according to the invention may be used on any sample type. The ease of the method combined with the high specificity and the absence of cross reactivity with other components of the sample, make the method especially suited for direct analysis of complex samples without any preceding purification step. Accordingly the method may advantageously be performed on samples selected from the group consisting of blood, urine, milk, tears, faeces, sperm, cerebrospinal fluid, nasal secrete, food, feed and mixtures, dilutions, or extracts thereof. More preferably the sample is selected from group consisting of blood, urine, milk, food and feed and mixtures, dilutions, or extracts thereof.

According to an especially preferred embodiment, the measurement of hydrophobic CoA esters is performed directly on blood or serum samples and dilutions or extracts thereof. More preferably this method comprises the determination of total lipids and/or free fatty acids in the blood or serum.

It is also envisages that the method according to the invention may be useful for measuring the level of hydrophobic CoA esters or the measurement of lipids and/or fatty acids in milk and dilutions or extracts thereof.

Due to the methods simplicity, it is also envisaged that the method according to the invention will be used for one step measurement of lipids and/or fatty acids in samples comprising food and dilutions or extracts thereof as well as in samples comprising feed and dilutions or extracts thereof.

The sample may also comprises urine and dilutions or extracts thereof. The presence of free fatty acids in the urine may be indicative of various diseases, among them the deficiency known as "mitochondrial medium chain acyl CoA dehydrogynase deficiency", which results in the presence of dicarboxylic acids of 8 of 12 carbon atoms in the urine.

The inventors have also determined that the constructs according to the invention are especially useful for determining the insulin sensitivity and the rate of lipolysis by adipocytes. Thereby, the constructs and the method may be used for early diagnosis of diabetes. Through an early diagnosis of diabetes, diet and diabetic treatment may be initiated early, and the occurrence of symptoms of diabetes such as blindness, macrovascular diseases such as generalised arteriosclerosis, hypertension, myocardial infarction, stroke, or microvascular diseases such as retinopathy or nephropathy, or neuropathy may be avoided or delayed. Avoidance and/or postponement of these symptoms have profound implications for the individuals suffering of diabetes and also results in enormous savings on public healthcare.

Assay kits

The construct and the method according to the invention may advantageously be combined in a kit for determination of the concentration of hydrophobic CoA esters in a sample. According to one aspect of the assay kit, the reagents may be loaded into a multiwell dish to which the sample is added and the assay performed. The detection may subsequently be performed in a multi-well reader.

In its simplest form, the assay kit is adapted for determination of the concentration of hydrophobic Co-A esters. In order to be useful for the determination of free fatty acids the kit may further comprise an acyl-Coenzyme A synthetase, coenzyme A, adenosinetriphosphate, Mg^{++}, an antioxidant, and buffer. If the thioesterification is carried out in the presence of a construct according to the invention, there may be no need for pyrophosphatase to drive the thioesterification reaction. If the thioesterification is carried out spatially separate from the construct according to the

invention, pyrophosphatase may advantageously be added to drive the thioesterification.

The kit according to the invention may also be adapted for determination of total lipids in which case it preferably comprises a lipase, and buffer to hydrolyse the triacylglycerides. For the determination of phospholipids the kit may comprise a phospholipase such as phospholipase A1 and/or A2, and buffer.

All the compounds used for the kits according to the invention may advantageously be freeze dried.

In some cases, especially those where the construct is not located in the sample compartment, it may be advantageous to add albumin to the kit. The presence of albumin ensures that free fatty acids and/or hydrophobic Co-A esters do not bind to the surfaces of the sample compartment. The albumin furthermore may be used for carrying the hydrophobic Co-A esters through a wick to immobilised constructs according to the invention.

The above described kits may either comprise a kit, wherein essentially all reagents (including the constructs according to the invention) are added to the sample compartment before addition of the sample.

Alternatively the assay kit may comprise at least one construct according to the invention, being immobilised on a solid support such as an extended solid phase. Such kits are known in the art under several names such as "lateral flow devices", or dip sticks. Illustrative and not limiting examples of suitable lateral flow devices that may be used in accordance with the present invention include those described in US 5,686,315 (PRONOVOST), US 4,943,522 (EISINGER et al), US 4,703,017 (BECTON DICKINSON) US 4,855,240 (BECTON DICKINSON), US 5,798,273 (BECTON DICKINSON). The extended solid phase is preferably of a type that allows a liquid sample comprising an analyte to diffuse through it without substantially binding the analytes or lowering the rate of movement of the analyte through the porous solid phase.

The extended solid phase may be in the shape of a dipstick, which may be dipped into a liquid sample, or it may have on it a sample compartment for applying a volume of sample, preferably a pre-determined amount of liquid sample. The kit may thus comprise a sample compartment and in another location a read out area in which constructs according to the invention are immobilised to the porous support phase and provide a signal when bound to hydrophobic CoA esters. The kit may be comprised in a housing with a hole for application of the sample into the sample compartment and a window for the read out area. After application of sample to the sample compartment, liquid sample moves through the porous solid support past the read out area to the end of the kit. The porous material may be any material to which the constructs can be linked, and which does not interfere with the assay such as through binding of free fatty acids, lipids or hydrophobic CoA esters. One suitable material may be nylon or nitrocellulose paper.

When the construct is immobilised, the sample may be added to the sample compartment, where it is optionally subjected to lipase and/or phospholipase and/or acyl-CoA ligase. The sample compartment advantageously also comprises albumin. After pre-treatment of the sample has been performed, the sample may be allowed to move via a wick to the immobilised construct. When pre-treatment is carried out in a sample compartment connected to the porous solid support, the kit preferably comprises means to seal the sample compartment from the porous solid support in order to avoid movement of sample through the porous support before the pre-treatment steps are concluded.

However, the pre-treatment may also be performed in another location such as in a test tube in order to avoid movement of liquid sample through the porous solid support before the pre-treatment steps are concluded.

As the liquid front reaches the immobilised constructs, the hydrophobic-CoA esters will be bound to the immobilised construct and the detection may be performed. Advantageously, the hydrophobicl-CoA esters are bound to albumin as they diffuse through the wick to the immobilised construct. As the affinity of the constructs according to the invention to is much higher than the affinity of albumin, albumin will deliver the CoA esters to the constructs.

The kits wherein the construct is immobilised may comprise constructs which are immobilised in at least two different places, such as at least 3, for example at least 4 such as at least 5 different spaces. In the case, where the constructs are identical, this embodiment is useful for rapid, one-step determination of the concentration of hydrophobic CoA esters or free fatty acids or lipids in a sample. A pre-determined amount of construct according to the invention, capable of binding a pre-determined amount of hydrophobic CoA esters is immobilised in two, three, four, five or more spaces on a stick. A predetermined amount of sample is added to the end of the stick after appropriate pre-treatment. As capillary forces move the liquid sample past the immobilised constructs, a pre-determined amount of hydrophobic CoA esters is bound to the immobilised constructs causing a change in the signal emitted from the constructs. As the liquid front has moved past all locations of immobilised construct the signals are detected. The larger the amount of hydrophobic CoA esters in the sample the more of the locations of construct will emit a signal indicative of bound CoA esters.

The kit according to the invention, may also comprise more than one construct such as a second hydrophobic-Coenzyme A ester binding construct, or at least a third construct, such as at least a third and a fourth construct, for example at least a third, a fourth and a fifth construct. It is to be understood that these constructs have a high binding affinity for different species of CoA ester or for a different group of CoA esters. By allowing a liquid sample to pass the immobilised constructs, different CoA esters will bind to different constructs. Upon detection, the presence of several species or groups of species may be detected. Through measurement of the intensity of the signals, the relative amount of different species and/or groups of species may be determined. Preferably each construct has a K_D with respect to at least one species or a group of species of hydrophobic Coenzyme A esters, which is substantially lower than the K_D of the other construct(s) with respect to this species or group of species.

According to a preferred embodiment of the invention, substantially lower may be 10 times lower, more preferably 100 times lower.

As a non-limiting example a kit according to the invention may comprise the first construct being a fluorescence acyl-CoA sensor 1 (FACS 1) and a second construct being a fluorescence acyl-CoA sensor 2 (FACS 2).

Coding sequences/expression vectors

The heterologous peptide comprised in the construct according to the invention may conveniently be manufactured using recombinant techniques. The invention therefore also features a nucleotide sequence encoding this heterologous peptide. Recombinant techniques for preparing nucleotide sequences are well known to the skilled practitioner.

The nucleotide sequence may be inserted into an expression vector, which is used for transformation of a cell. Eventually the cell comprises the nucleotide sequence encoding the peptide part of the construct under the control of a suitable promoter. The construct may be manufactured by the cell, harvested and optionally purified further prior to addition of the signal moiety.

Alternatively the peptide may be manufactured using well known chemical synthesis methods.

Example 1 Site-directed mutagenesis using the QuikChange (Stratgene)

Template (50 ng of Bov-ACBP in pET3a) was incubated in *Pfu* Turbo reaction buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ 0.1 % Triton X-100, 0.1 mg/ml BSA) supplemented with 0.25 mM dNTP, 125 ng of each mutagenic primer and 2.5 U *Pfu* Turbo polymerase in final volume 50 ml. The reaction was cycled using the following parameters: 95 °C/ 30 s, 55 °C/ 2 min, 68 °C/ 10 min for 16 cycles. Subsequently, the reaction was placed at 37 °C and 10 U of *DpnI* was added to remove the parental DNA and incubated for at least 1 hour. Finally the DNA was transformed into competent DH5a cells. Ampecilin resistant transformants were selected and plasmids were purified using the plasmid kit from Qiagen. Plasmids were sequenced using the CEQ DTCS kit (Beckman) as described by the manufacturer. Plasmids containing the desired mutation were

transformed into BL21(DE3)p*LysS* and protein was induced and purified as described previously.

The recombinant Met24_Cys24-, Phe49_Cys49- and Ala53_cys_53-bovine ACBP were fluorescently labelled with 6-bromoacetyl-2-dimethylaminonaphthalene (Badan, Molecular Probes). Badan was used because of its sensitivity to polarity of its environments and which was expected to make it particularly sensitive to interaction of hydrophobic-CoA esters with ACBP and because Badan is capable of covalent modification of protein amino acid residues. To carry out the reaction 1.2 mole excess badan over ACBP was added over 10 min by continuous infusion from a 20 mM stock solution of Badan in dimethylformamide, to a 1 mg/ml solution of Met24_cys24-, Phe49_cys49 or Ala53_Cys53-bovine ACBP in 50 mM tris/HCL pH 7.2. Incubation was continued for 15 min and the reaction was stopped by addition of excess DTT. Unreacted badan and badan side reaction products were removed by passing the reaction product over a 1 ml Lipidex-1000 column. The resulting derivatised protein was shown to have a stochiometry of 1 badan per mole protein by electrospray mass spectrometry. The localisation of the badan derivatised amino acid was confirmed by tryptic digestion and separation of the tryptic peptides by reverse phase HPLC using water /acetonitrile /TFA solvent system followed by mass determination and sequencing of the flourescently labelled peptide.

Example 2: Dissociation constant of the construct/ligand complex

Quantitative determination of binding affinities (K_D) were performed by isothermal titration micro calorimetry as previously described (Faergeman et. al., 1996; Biochemistry 35, 14118-14126). Qualitative evaluation of relative binding affinities were determined by isoelectrical focusing using the Pharmacia Fast Gel system according to the prescriptions given by the manufacture. Fluorescence emission changes induced by acyl-CoA binding to badan modified protein were determined as follows: 5µl portions of acyl-CoAs were added from stock solutions disolved in binding buffer, (10mM Hepes, 150mM NaCL, 1 mM $NaHPO_4$, pH 7,4) containing 3.4 µM Badan derivatized protein to a 1 ml 3.4 µM solution of the Badan derivatized ACBP in the same buffer. The fluorescence emission was measured on a SPEX FLOUROLOG (Industries Inc, Edison NJ, USA) with excitation at 400 nm and emission scan from 400 nm to 550 nm. The concentration of acyl-CoA in the

aqueous phase was determined from the fluorescence emission sensitivities at 495 and 470 nm repectively essentially by the method described by (Grynkiewickz et. al.,1985, J. Biol. Chem. 260, 3440-3450) according to which:

$[acyl\text{-}CoA]_{free} = K_D((F-F_{min})/(F_{max}-F)$

Where F is the measured fluorescence in the solution and F_{min} the fluorescence in the absence of ligand and F_{max} the fluorescence in the presence of saturating ligand concentration.

The exclusive binding of hydrophobic-CoA esters by ACBP is determined by specific recognition of the CoA head group (Kragelund, et. al.,1993; J Mol Biol, 230(4):1260-1277) CoA it self is bound with low affinity (K_D = 2 µM) with increasing acyl-chain length the affinity increases (K_D ~ 1-2 nM) up to 22 carbons after which the binding affinities drop dramatically (Faergeman et. al., 1996; Biochemistry 35, 14118-14126; Rosendal, et. al.,1993; Biochem J. 290, 321-326; Robinson, C.V., 1996. J. Am. Chem. Soc., 118, 8646-8653). The mutated amino acid residues were chosen as residues which have been shown to interact with the acyl-chain of the bound acyl-CoA in the ACBP/acyl-CoA complex (Kragelund, et. al.,1993; J Mol Biol, 230(4):1260-1277). The primary structure of ACBP is highly conserved throughout eukaryote from *S. pompe* to man and the basic structure and binding properties is expected to be very similar in ACBP from all species (Kragelund, et. al., 1999, Biochim Biophys Acta. 1441, 150-61). The obtained reitsults with the Badan derivatised bovine ACBPs are therefore expected to be representative for ACBP from all species. We are presently making the Met24_Cys24- badan analog of Yeast and rat ACBP to confirm this.

Example 3: One step assay of FFA.

To demonstrate the ability of FACS1 to act as a sensor for determining the level of total free non-esterified fatty acids in biological fluids FACS1 (4 µM) was incubated in a reaction mixture containing: 100mM Tris/HCL pH 7.4, 1 mm DTT, 2 mM EDTA, 4mM $Mg(CL)_2$, 4 mM ATP, 60 µM CoA, 0.03 units/ml Acyl-CoA synthetase and 0.06 units/ml Pyrophosphatase at 37 °C for 30 min. The reaction was started by addition of human serum or free fatty acid standard bound to equimolar amounts of bovine

serum albumin. The results in fig 5 A and B show that the present invention makes it possible to determine the formed acyl-CoA in a one step reaction simultaneously with the formation of the acyl-CoA esters by the acyl-CoA synthetase direct in the reaction mixture. The use of FACS1 to determine the formed acyl-CoA esters increase the sensitivity of present methods and make it possible to determine FFA in less than one micro liter of serum (Fig 5). A total fatty acid method based on the FACS1 sensor will be of great value, it will simplify present assays and make it possible to measure total fatty acids in body fluid from even very small species and infant.

The standard curve in Figure 5A was prepared using the following mix of reagents:

M24C-BADAN	3µM
CoA	60µM
$MgCl_2$	4mM
EDTA	2 mM
AcylCoA synthetase	0.03 units/mL
Pyrophosphatase	0.06 units/ML
Tris/HCl, pH 7.4	100 mM

1 mL of the reaction mix was added to different amounts of 50 µM palmitic acid (dissolved in 100 mM Tris/HCl, 50 µM bovine serum albumin) to a final concentration of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 6.0 µM. The mixture was incubated for 30 min at 37°C and then the sample was excitated at 400 nm and the emission was measured at 470 nm.

The curve in Figure 5B was prepared using the following mix of reagents:

M24C-BADAN	4µM
CoA	60µM
$MgCl_2$	4mM
EDTA	2 mM
AcylCoA synthetase	0.03 units/mL
Pyrophosphatase	0.06 units/ML
Tris/HCl, pH 7.4	100 mM

1 mL of the reaction mix was added to different amounts of plasma 0, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 µL. The mixture was incubated for 30 min at 37°C and then the sample was excitated at 400 nm and the emission was measured at 470 nm.

In order to test the ability of FACS1 to function as a sensor of free unbound acyl-CoA native bovine ACBP (5.58 µM) was titrated with dodecanoyl-CoA (C12-CoA) in the presence of FACS1 (0.58 µM) and the free C12-CoA concentrations were calculated from fluorescence emission changed of FACS1 induced by C12-CoA titration. The K_D for C12-CoA binding to M24C-badan ACBP, used in the calculation of free acyl-CoA concentration from fluorescence measurements, was determined to 40nM using isotermal titration micro calorimetry . This concentration was compared with concentrations calculation from the predetermined K_D for C12-CoA binding to native bovine ACBP (Færgeman et al. Biochemistry. 1996 Nov 12;35(45):14118-26. The results in fig 6 demonstrate that there is a good agreement between the calculated and the measured free acyl-CoA concentration measured using FACS1. This demonstrate that FACS1 functions as a sensor for measuring free acyl-CoA concentration.

The data presented herein demonstrate that FACS1 and FACS2 are highly specific and extremely sensitive probes for free non-esterified fatty after conversion to acyl-CoA esters and free C8- to C20-acyl-CoA esters in aqueous solution in the low nM range.

Claims

1. A method for determination of the concentration of free unbound hydrophobic Coenzyme A ester in a sample comprising the steps of
   - i) providing a hydrophobic Coenzyme A binding construct exhibiting a first signal when unbound and exhibiting a measurably different second signal when bound to a hydrophobic Coenzyme A ester,
   - ii) contacting the sample with the labelled hydrophobic Coenzyme A binding construct,
   - iii) allowing at least one species of unbound free hydrophobic Coenzyme A ester to bind to the hydrophobic Coenzyme A binding construct forming a complex comprising a hydrophobic Coenzyme A ester and the hydrophobic Coenzyme A binding construct,
   - iv) detecting a signal from the complex,
   - v) correlating the signal to the concentration of the at least one species of hydrophobic Coenzyme A ester in the sample.

2. The method according to any 1, whereby the heterologous peptide comprises an acyl-CoenzymeA binding protein, a variant or functional equivalent thereof.

3. The method according to claim 2, whereby the acyl-Coenzyme A binding protein comprises an amino acid sequence from the sequences of Figure 1 (SEQ ID NO 1 TO 30) a variant or functional equivalent thereof.

4. The method according to claim 3, whereby the variant or functional equivalent has at least 30% sequence identity to one of the sequences of Figure 1(SEQ ID NO 1 TO 30), such as at least 40 % sequence identity, for example at least 50 % sequence identity, such as at least 55% sequence identify, for example at least 60% sequence identity, such as at least 65 % sequence identity, for example at least 70 % sequence identity, such as at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, such as at least 91 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such

as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity.

5. The method according to any of the preceding claims, whereby the heterologous peptide comprises an acyl-CoenzymeA binding domain.

6. The method according to any of the preceding claims, whereby the heterologous peptide comprises bovine ACBP, a variant or functional equivalent thereof.

7. The method according to any of the preceding claims, whereby the heterologous peptide comprises a cystein or lysin residue for binding the signal moiety.

8. The method according to any of the preceding claims, whereby one native amino acid in the heterologous peptide has been substituted by a cystein residue for binding the signal moiety.

9. The method according to any of the preceding claims, whereby one native amino acid in the heterologous peptide has been substituted by a lysin residue for binding the signal moiety.

10. The method according to claim 7 to 9, whereby the residue is selected from the amino acid residues aligning the acyl Coenzyme A binding domain.

11. The method according to claim 7 to 9, whereby the residue is selected from the amino acid residues having van der Waals' contact with a bound hydrophobic Coenzyme A ester.

12. The method according to claim 7 to 9, whereby the residue is selected from the amino acid residues being within 5 Å from a bound hydrophobic Coenzyme A ester.

13. The method according to claim 7 to 9, whereby the residue is selected from the amino acid residues making up the α-helices of the heterologous peptide.

14. The method according to any of the preceding claims, whereby the heterologous peptide comprises a first α-helix, A1, a second α-helix, A2, a third α-helix, A3 and a fourth α-helix, A4.

15. The method according to claim 14, whereby the heterologous peptide further comprises a N-terminal peptide linked to A1, an A1-A2 linking peptide, an A2-A3 linking peptide, an A3-A4 linking peptide, and optionally an N-terminal peptide linked to the C-terminal of A4.

16. The method according to claim 5, whereby the N-teminal peptide comprises at least 3 amino acids, such as at least 10, for example at least 20, such as at least 30, 40, or 50 amino acids, for example at least 100 more amino acids; the A1-A2 linking peptide comprises 5 to 9 amino acids; the A2-A3 linking peptide comprises 14 to 15 amino acids; the A3-A4 linking peptide comprises 2 peptides; and the optional C-terminal peptide comprises at least 1, 2 or 3 amino acids, such as at least 10, for example at least 20, such as at least 30, for example at least 50, such as at least 75 or 100, for example at least 200, such as at least 300, 400 or 500 amino acids, for example at least 750 or 1000.

17. The method according to claim 8 and 16, whereby the substituted amino acid is selected from the group consisting of amino acids number 3 and 4 of the A1-A2 linking peptide; amino acids number 13 and 14 of the A2-A3 linker.

18. The method according to claim 14, whereby A1 comprises 12 amino acid residues; A2 comprises 16 amino acid residues; A3 comprises 12 amino acid residues; and A4 comprises 20 amino acid residues.

19. The method according to claim 8 and 18, whereby the substituted amino acid residue is selected from the group consisting of amino acids number 6, 9, 10, and 12 of A1; amino acids number 4, 5, 7, 8, 11, and 12 of A2; amino acids number 3 and 4 of A3; amino acid number 9 of A4.

20. The method according to any of the preceding claims, whereby the heterologous peptide comprises the bovine ACBP and the native amino acid being replaced by a cystein residue is selected from the group consisting of Phe-49, Met24,

Leu-25, Ala-53, Asp-21, Lys-50, Lys-54, Lys-18, pro-19, Ala-9, Tyr-31, Lys-32, Tyr-28, Tyr-73, Val-12, Lys-13, Leu-15; Ile-27; more preferably whereby the native amino acid is selected from the group consisting of Met-24 and Ala-53.

21. The method according to any of the preceding claims, whereby the N-terminal peptide comprises an affinity tag such as a poly His tag, more preferably a polyHis tag of at least 5 residues, such as at least 6 residues, for example at least 10 residues, such as at least 15 residues, for example at least 20 residues.

22. The method according to any of the preceding claims, whereby the complex formed during step iii) has a K_D below 2 µM, such as below 1.5 µM, for example below 1.0 µM, preferably below 500 nM, more preferably below 200 nM such as below 100 nM, for example below 90 nM, such as below 80 nM, for example below 70 nM, such as below 60 nM, for example below 50 nM, such as below 40 nM, for example below 30 nM, such as below 20 nM, for example below 15 nM, such as below 10 nM, for example below 8 nM, such as below 7 nM, for example below 6 nM, such as below 5 nM, for example below 4 nM, such as below 3 nM, for example below 2 nM, such as below 1 nM.

23. The method according to claim 22, whereby the complex formed during step iii) has a K_D below 2 µM, such as below 1.5 µM, for example below 1.0 µM, preferably below 500 nM, more preferably below 200 nM, such as below 100 nM, for example below 90 nM, such as below 80 nM, for example below 70 nM, such as below 60 nM, for example below 50 nM, such as below 40 nM, for example below 30 nM, such as below 20 nM, for example below 15 nM, such as below 10 nM, for example below 8 nM, such as below 7 nM, for example below 6 nM, such as below 5 nM, for example below 4 nM, such as below 3 nM, for example below 2 nM, such as below 1 nM and a higher K_D with respect to other species of hydrophobic Coenzyme A esters.

24. The method according to claim 23, whereby the one species of hydrophobic Coenzyme A ester is selected from the group consisting of acyl Coenzyme A esters having a C2 acyl group, a C4 acyl group, a C6 acyl group, a C8 acyl group, a C10 acyl group, a C12 acyl group, a C14 acyl group, a C16 acyl group, a C18 acyl group, a C20 acyl group, a C22 acyl group, a C24 acyl group, a C26

acyl group, a saturated acyl group, a mono-unsaturated acyl group, a polyunsaturated acyl group, an acyl group comprising a cis double bond, an acyl group comprising a trans double bond, an acyl group comprising a ring structure, an acyl group comprising a side chain.

25. The method according to any of the preceding claims, whereby the signal comprises a fluorescence signal.

26. The method according to any of the preceding claims, whereby a signal comprises a chromogenic signal.

27. The method according to any of the preceding claims, whereby a signal comprises a chemiluminiscense signal.

28. The method according to any of the preceding claims, whereby a signal is a photoluminiscense signal.

29. The method according to any of the preceding claims, whereby the detected signal is the second signal.

30. The method according to any of the preceding claims, whereby the first signal is essentially zero.

31. The method according any of the preceding claims, whereby the detected signal is the difference between the first and the second signal.

32. The method according to any of the preceding claims, whereby the detected signal is essentially proportional to the amount of hydrophobic Coenzyme A ester in the sample.

33. The method according to claim 32, whereby the detected signal is essentially proportional to the amount of at least one species of Coenzyme A ester in the sample.

34. The method according to claim 33, whereby the at least one species of Coenzyme A ester comprises a species selected from the group consisting of Coenzyme A esters with a C2 acyl group, a C4 acyl group, a C6 acyl group, a C8 acyl group a C10 acyl group, a C12 acyl group, a C14 acyl group, a C16 acyl group, a C18 acyl group, a C20 acyl group, a C22 acyl group, a C24 acyl group, a C26 acyl group, a saturated acyl group, a mono-unsaturated acyl group, a polyunsaturated acyl group, an acyl group comprising a cis double bond, an acyl group comprising a trans double bond, an acyl group comprising a ring structure, an acyl group comprising a side chain.

35. The method according to any of the preceding claims, whereby the detected signal from a first species of hydrophobic Coenzyme A ester is essentially 0 and the detected signal from a second species of hydrophobic Coenzyme A is essentially proportional to the amount of said second species in the sample.

36. The method according to claim 35, whereby the first species comprises a saturated species and the second species comprises an unsaturated species or vice versa.

37. The method according to claim 35, whereby the first species comprises a mono-unsaturated species and the second species comprises a poly-unsaturated species or vice versa.

38. The method according to claim 35, whereby the first species comprises a species with a cis-double bond and the second species comprises a trans-double bond or vice versa.

39. The method according to claim 35, whereby the first species comprises a double bond and the second species comprises a double bond in another position.

40. The method according to any of the preceding claims, whereby the detected signal is essentially proportional to the amount of a group of hydrophobic Coenzyme A esters in the sample.

41. The method according to claim 40, whereby the group comprises Coenzyme A esters with a C2-C6 acyl group.

42. The method according to claim 40, whereby the group comprises Coenzyme A esters with a C8-C12 acyl group.

43. The method according to claim 40, whereby the group comprises Coenzyme A esters with a C12-C16 acyl group.

44. The method according to claim 40, whereby the group comprises Coenzyme A esters with a C16-C20 acyl group.

45. The method according to claim 40, whereby the group comprises Coenzyme A esters with a C12-C20 acyl group.

46. The method according to claim 40, whereby the group comprises Coenzyme A esters with a C22-C24 group.

47. The method according to claim 40, whereby the group comprises Coenzyme A esters with a C6-C10 acyl group, or a C10-C14 acyl group, or a C14-C18 acyl gruop, or a C18-C22 acyl group, or a C4-C8 acyl group, or a C8-C16 acyl group, or a C4-C12 acyl group, or an acyl group comprising more than 20 carbon atoms.

48. The method according to any of the preceding claims, further comprising a step prior to step ii) wherein hydrophobic acids in the sample are converted to hydrophobic Coenzyme A esters.

49. The method according to claim 48, whereby the conversion is catalysed by acyl Coenzyme A ligase.

50. The method according to claim 48 or 49, further comprising a prior step wherein triacylglycerides in the sample are converted to glycerol and free fatty acids.

51. The method according to claim 50, whereby the conversion is catalysed by lipase.

52. The method according to claim 50, whereby the conversion comprises acid or basic ester hydrolysis.

53. The method according to claim 48 or 49, further comprising a prior step wherein phospholipids in the sample are converted to glycerol and free fatty acids.

54. The method according to claim 53, whereby the conversion is catalysed by phospholipase A1 and/or phospholipase A2.

55. The method according to claim 53, whereby the conversion comprises acid or basic ester hydrolysis.

56. The method according to any of the preceding claims, whereby the sample is selected from the group consisting of blood, urine, milk, tears, faeces, sperm, cerebrospinal fluid, nasal secrete, food, feed and mixtures, dilutions, or extracts thereof.

57. The method according to any of the preceding claims, whereby the sample is selected from the group consisting of blood, urine, milk, food and feed and mixtures, dilutions, or extracts thereof.

58. The method according to any of the preceding claims, whereby the sample comprises blood and dilutions or extracts thereof.

59. The method according to any of the preceding claims, whereby the sample comprises milk and dilutions or extracts thereof.

60. The method according to any of the preceding claims, whereby the sample comprises food and dilutions or extracts thereof.

61. The method according to any of the preceding claims, whereby the sample comprises feed and dilutions or extracts thereof.

62. The method according to any of the preceding claims, whereby the sample comprises urine and dilutions or extracts thereof.

63. The method according to any of the preceding claims, whereby the complex formed during step iii) has a K_D below 2 µM, such as below 1.5 µM, such as below 1.0 µM preferably below 500 nM, more preferably below 200 nM, such as below 100 nM, for example below 90 nM, such as below 80 nM, for example below 70 nM, such as below 60 nM, for example below 50 nM, such as below 40 nM, for example below 30 nM, such as below 20 nM, for example below 15 nM, such as below 10 nM, for example below 8 nM, such as below 7 nM, for example below 6 nM, such as below 5 nM, for example below 4 nM, such as below 3 nM, for example below 2 nM, such as below 1 nM.

64. A construct for binding hydrophobic Coenzyme A ester comprising

i) a heterologous peptide capable of binding at least one species of hydrophobic Coenzyme A ester,

ii) a signal moiety.

65. The construct according to claim 64, wherein the signal moiety comprises a fluorescent moiety.

66. The construct according to claim 64 or 65, wherein the signal moiety comprises a chemiluminescent moiety.

67. The construct according to any of claims 64 to 66, wherein the signal moiety comprises a photoluminescent moiety.

68. The construct according to any of claims 64 to 67, wherein the signal moiety comprises a chromogenic moiety.

69. The construct according to any of claims 64 to 68, wherein the signal moiety exhibits a first signal when the construct is unbound and a measurably different second signal when the construct is bound to a hydrophobic-Conenzyme A ester.

70. The construct according to any of claims 64 to 69, wherein the signal moiety comprises (6-bromoacetyl-2-dimethylaminonaphtalene) BADAN.

71. The construct according to claim 65, wherein the fluorescent moiety comprises a compound selected from the group consisting of acrylodan; 5-dimethylaminonaphtalene-1-sulfonyl aziridine (danzyl aziridine); 4-[N-[2-Iodoacetoxy)ethyl]-N-methylamino]-7-nitrobenz-2-oxa 1,3 diazole ester (IANBDE); 4-[N-[2-iodoacetoxy)ethyl]-N-methylamino]-7-nitrobenz-2-oxa 1,3 diazole amide (IANBDA); 6-acryloyl-2-dimtheylaminonaphtalene (acrylodan); N-(7-chlorobenz-2-oxa-1,3-diazyl-4-yl)sulfonyl morpholine; 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride); didansyl-L-cystine; N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide); 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F); 4-fluoro-7-nitrobenz-2-

oxa-1,3-diazole (NBD fluoride); 2-(4'-(iodoacetamido)anilino)naphtalene-6-sulfonic acid, sodium salt (IAANS); 5-(((2-iodoacetyl)amino)ethyl)amino)naphtalene-1-sulfonic acid (1,5-IAEDANS); 2-(4'-maleimidylanilino)naphtalene-6-sulfonic acid (MIANS); N-(1-pyreneethyl)iodoacetamide; N-(1-pyrene)iodoacetamide; N-(1-pyrene)maleimide; N-(1-pyrenemethyl)iodoacetamide (PMIA amide); 1-pyrenemethyl iodoacetate (PMIA ester); N-(1-pyrenepropyl)iodoacetamide); 1-(2,3-epoxypropyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium trifluoromethanesulfonate (PyMPO epoxide); erythrosin-5-iodoacetamide; fluorescein-5-maleimide; 5-iodoacetamidofluorescein (5-IAF); 6-iodoacetamidofluorescein (6-IAF); 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate (PyMPO maleimide); Oregon Green™ 488 iodoacetamide "mixed isomers"; tetramethylrhodamine-5-iodoacetamide (5-TMRIA) "single isomer"; tetramethylrhodamine-5-maleimide "single isomer"; tetramethylrhodamine-6-maleimide "single isomer"; Texas Red® C_5 bromoacetamide; Texas Red® C_2 maleimide;

72. The construct according to any of claims 64 to 71, further comprising a second signal moiety.

73. The construct according to claim 72, wherein the second signal moiety is selected from the group of claim 71.

74. The construct according to any of claims 64 to 73, wherein the signal moiety is bound to a cystein or a lysin residue comprised in the heterologous peptide.

75. The construct according to any of claims 64 to 74, wherein the heterologous peptide comprises an acyl-CoenzymeA binding protein, a variant or functional equivalent thereof.

76. The construct according to claim 74, wherein the acyl-Coenzyme A binding protein comprises an amino acid sequence from the sequences of Figure 1 (SEQ ID NO 1 TO 30) a variant or functional equivalent thereof.

77. The construct according to claim 76, wherein the variant or functional equivalent has at least 30% sequence identity to one of the sequences of Figure 1, such as at least 40 % sequence identity, for example at least 50% sequence identity, such as at least 55% sequence identify, for example at least 60% sequence identity, such as at least 65 % sequence identity, for example at least 70 % sequence identity, such as at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, such as at least 91 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity.

78. The construct according to any of claims 64 to 77, wherein the heterologous peptide comprises an acyl-CoenzymeA binding domain.

79. The construct according to any of claims 64 to 78, wherein the heterologous peptide comprises bovine ACBP, a variant or functional equivalent thereof.

80. The construct according to claim 64 and 74, wherein the heterologous peptide comprises a cystein residue for binding the signal moiety.

81. The construct according to claim 64 and 74, wherein one native amino acid in the heterologous peptide has been substituted by a cystein residue for binding the signal moiety.

82. The construct according to claim 64 and 74, wherein one native amino acid in the heterologous peptide has been substituted by a lysin residue for binding the signal moiety.

83. The construct according to claim 70-74, wherein the residue is selected from the amino acid residues aligning the acyl Coenzyme A binding domain.

84. The construct according to claim 70-74, wherein the residue is selected from the amino acid residues having van der Waals' contact with a bound hydrophobic Coenzyme A ester.

85. The construct according to claim 70-74, wherein the residue is selected from the amino acid residues being within 5 Å from a bound hydrophobic Coenzyme A ester.

86. The construct according to claim 70-74, wherein the residue is selected from the amino acid residues making up the α-helices of the heterologous peptide.

87. The construct according to any of claims 64 to 86, wherein heterologous peptide comprises a first α-helix, A1, a second α-helix, A2, a third α-helix, A3 and a fourth α-helix, A4.

88. The construct according to claim 87, wherein the heterologous peptide further comprises a N-terminal peptide linked to A1, an A1-A2 linking peptide, an A2-A3 linking peptide, an A3-A4 linking peptide, and optionally an N-terminal peptide linked to the C-terminal of A4.

89. The construct according to claim 88, wherein the N-teminal peptide comprises at least 3 amino acids, such as at least 10, for example at least 20, such as at least 30, 40, or 50 amino acids, for example at least 100 more amino acids; the A1-A2 linking peptide comprises 5 to 9 amino acids; the A2-A3 linking peptide comprises 14 to 15 amino acids; the A3-A4 linking peptide comprises 2 peptides; and the optional C-terminal peptide comprises at least 1, 2 or 3 amino acids, such as at least 10, for example at least 20, such as at least 30, for example at least 50, such as at least 75 or 100, for example at least 200, such as at least 300, 400 or 500 amino acids, for example at least 750 or 1000.

90. The construct according to claim 81 and 89, wherein the substituted amino acid is selected from the group consisting of amino acids number 3 and 4 of the A1-A2 linking peptide; amino acids number 13 and 14 of the A2-A3 linker.

91. The construct according to claim 87, wherein A1 comprises 12 amino acid residues; A2 comprises 16 amino acid residues; A3 comprises 12 amino acid residues; and A4 comprises 20 amino acid residues.

92. The construct according to claim 81 and 91, wherein the substituted amino acid residue is selected from the group consisting of amino acids number 6, 9, 10, and 12 of A1; amino acids number 4, 5, 7, 8, 11, and 12 of A2; amino acids number 3 and 4 of A3; amino acid number 9 of A4.

93. The construct according to the claim 81, wherein the heterologous peptide comprises the bovine ACBP and the native amino acid being replaced by a cystein residue is selected from the group consisting of Phe-49, Met24, Leu-25, Ala-53, Asp-21, Lys-50, Lys-54, Lys-18, pro-19, Ala-9, Tyr-31, Lys-32, Tyr-28, Tyr-73, Val-12, Lys-13, Leu-15; Ile-27; more preferably wherein the native amino acid is selected from the group consisting of Met-24 and Ala-53.

94. The construct according to any of the preceding claims 64 to 93, wherein the N-terminal peptide comprises an affinity tag such as a poly His tag, more preferably a polyHis tag of at least 5 residues, such as at least 6 residues, for example at least 10 residues, such as at least 15 residues, for example at least 20 residues.

95. The construct according to any of claims 87 to 94, having a K_D with respect to at least one hydrophobic Coenzyme A ester below 2 µM, such as below 1.5 µM, for example below 1.0 µM, preferably below 500 nM, more preferably below 200 nM such as below 100 nM, for example below 90 nM, such as below 80 nM, for example below 70 nM, such as below 60 nM, for example below 50 nM, such as below 40 nM, for example below 30 nM, such as below 20 nM, for example below 15 nM, such as below 10 nM, for example below 8 nM, such as below 7 nM, for example below 6 nM, such as below 5 nM, for example below 4 nM, such as below 3 nM, for example below 2 nM, such as below 1 nM.

96. The construct according to claim 95, having a K_D with respect to one species of hydrophobic Coenzyme A ester below 2 µM, such as below 1.5 µM, for example below 1.0 µM, preferably below 500 nM, more preferably below 200 nM, such as

below 100 nM, for example below 90 nM, such as below 80 nM, for example below 70 nM, such as below 60 nM, for example below 50 nM, such as below 40 nM, for example below 30 nM, such as below 20 nM, for example below 15 nM, such as below 10 nM, for example below 8 nM, such as below 7 nM, for example below 6 nM, such as below 5 nM, for example below 4 nM, such as below 3 nM, for example below 2 nM, such as below 1 nM and a higher K_D with respect to other species of hydrophobic Coenzyme A esters.

97. The construct according to claim 96, wherein the one species of hydrophobic Coenzyme A ester is selected from the group consisting of acyl Coenzyme A esters having a C2 acyl group, a C4 acyl group, a C6 acyl group, a C8 acyl group, a C10 acyl group, a C12 acyl group, a C14 acyl group, a C16 acyl group, a C18 acyl group, a C20 acyl group, a C22 acyl group, a C24 acyl group, a C26 acyl group, a saturated acyl group, a mono-unsaturated acyl group, a polyunsaturated acyl group, an acyl group comprising a cis double bond, an acyl group comprising a trans double bond, an acyl group comprising a ring structure, an acyl group comprising a side chain.

98. A kit for detection of the concentration of a hydrophobic Coenzyme A ester in a sample comprising
    i) at least a first construct according to claims 64 to 97,
    ii) a sample compartment for application of the sample.

99. The kit according to claim 98, further comprising an acyl-Coenzyme A synthetase, coenzyme A, adenosinetriphosphate, Mg^{++}, an antioxidant, and buffer.

100. The kit according to claim 99, further comprising pyrophosphatase.

101. The kit according to claim 99 or 100, further comprising a lipase, and buffer.

102. The kit according to any of the preceding claims 99-101, further comprising a phospholipase such as phospholipase A1 and/or A2, and buffer.

103. The kit according to any of the preceding claims 99-102, further comprising an esterase specific for cholesterol esters.

104. The kit according to any of the preceding claims 98-103, further comprising albumin.

105. The kit according to any of claims 98 to 104, wherein compounds are freeze dried.

106. The kit according to any of the preceding claims 98-105, wherein the hydrophobic-Coenzyme A ester binding construct is immobilised.

107. The kit according to claim 106, wherein the construct is immobilised in at least two different places, such as at least 3, for example at least 4 such as at least 5 different spaces.

108. The kit according to any of the preceding claims 98-107, comprising a second hydrophobic-Coenzyme A ester binding construct according to claims 64 to 97.

109. The kit according to claim 108, further comprising at least a third construct, such as at least a third and a fourth construct, for example at least a third, a fourth and a fifth construct.

110. The kit according to any of claims 108 to 109, wherein each construct has a K_D with respect to at least one species or a group of species of hydrophobic Coenzyme A esters, which is substantially lower than the K_D of the other construct(s) with respect to this species or group of species.

111. The kit according to claim 110, wherein substantially lower is 10 times lower, preferably 100 times lower.

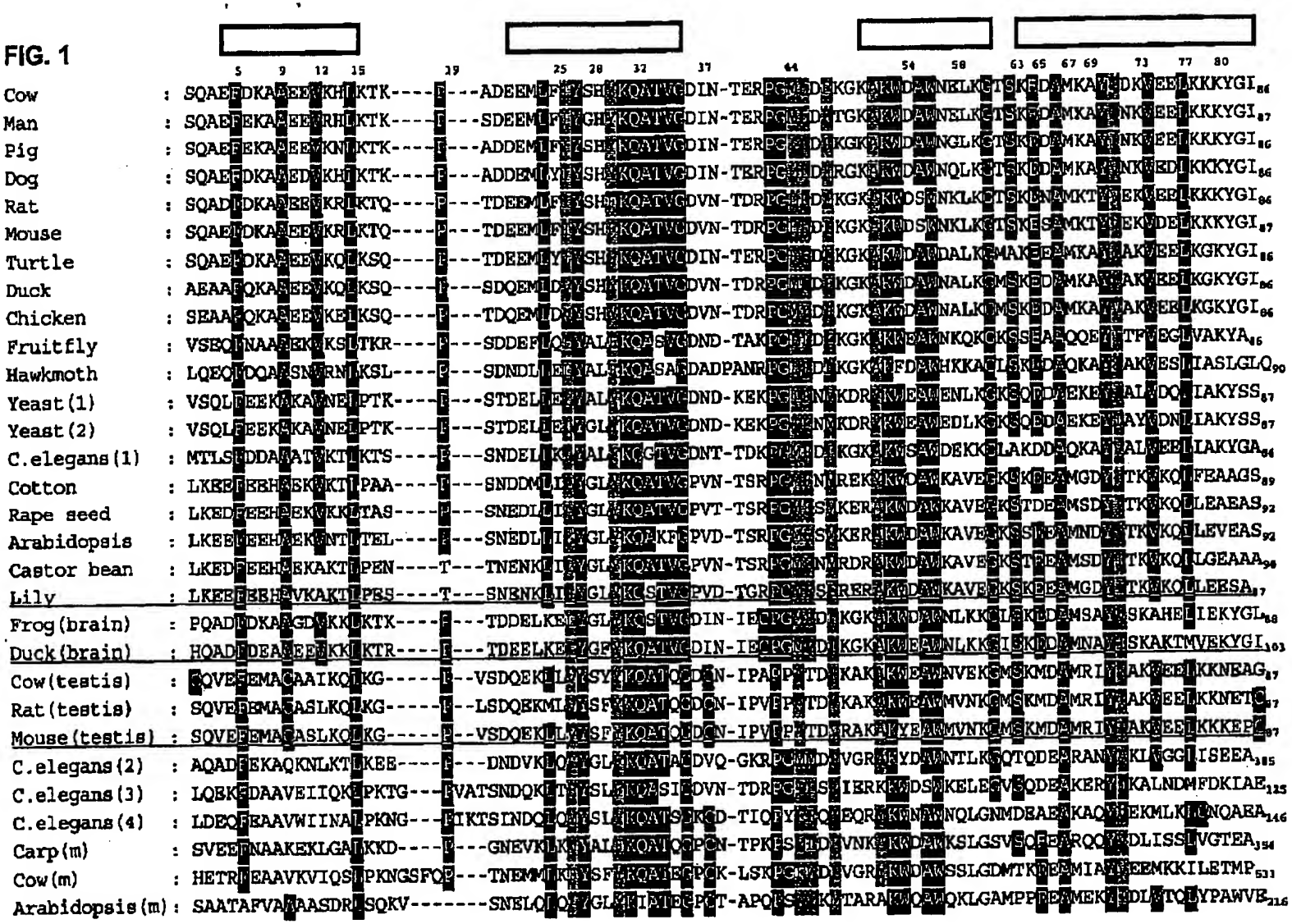
112. The kit according to claim 110, wherein the first construct is a fluorescence acyl-CoA sensor 1 (FACS 1) and the second construct is a fluorescence acyl-CoA sensor 2 (FACS 2).

113. A nucleotide sequence encoding the heterologous peptide according to claim 64 to 97.

114. An expression vector comprising the nucleotide sequence according to claim 113.

115. A cell comprising the nucleotide sequence according to claim 113.

FIG. 1



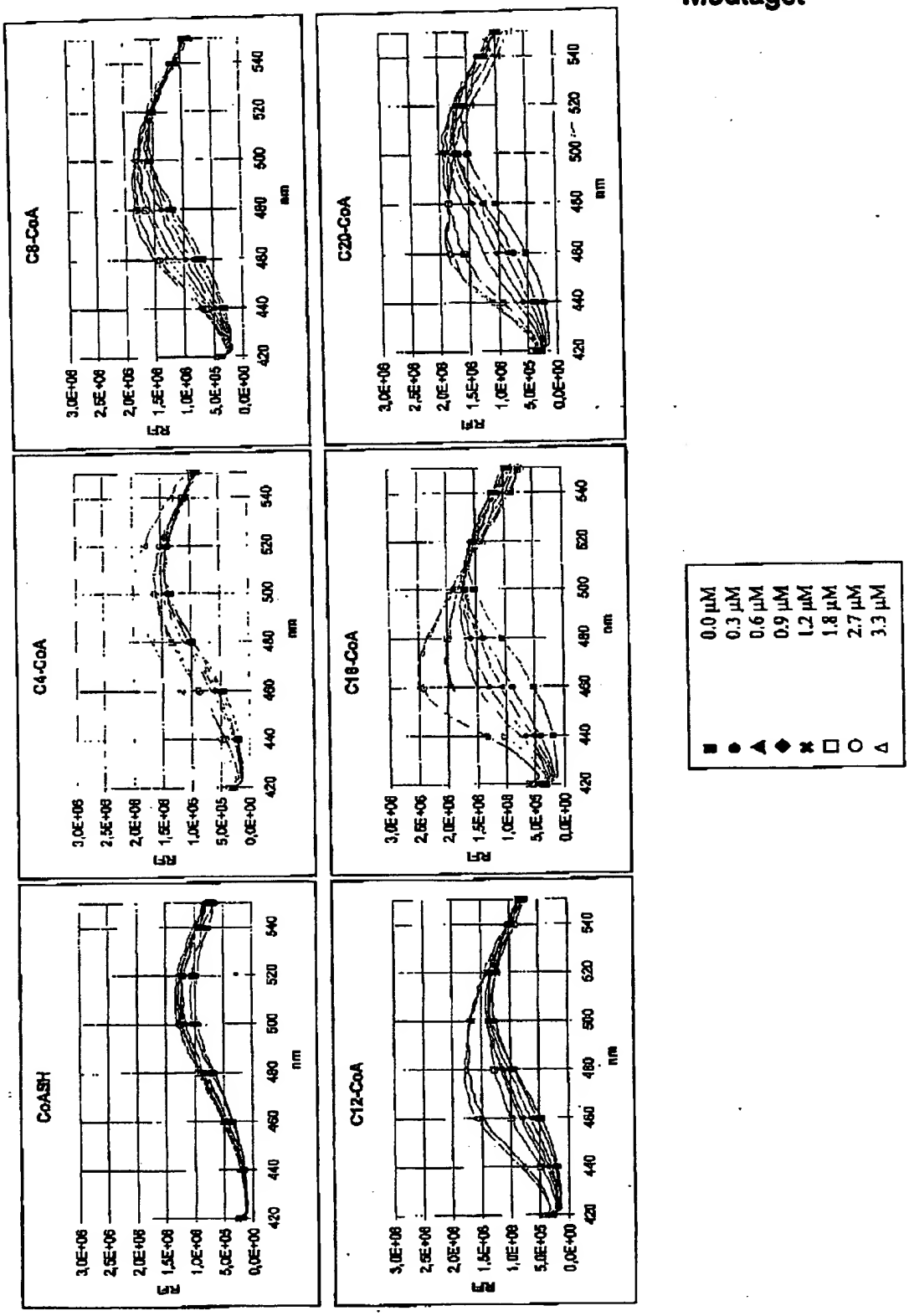


FIG. 2

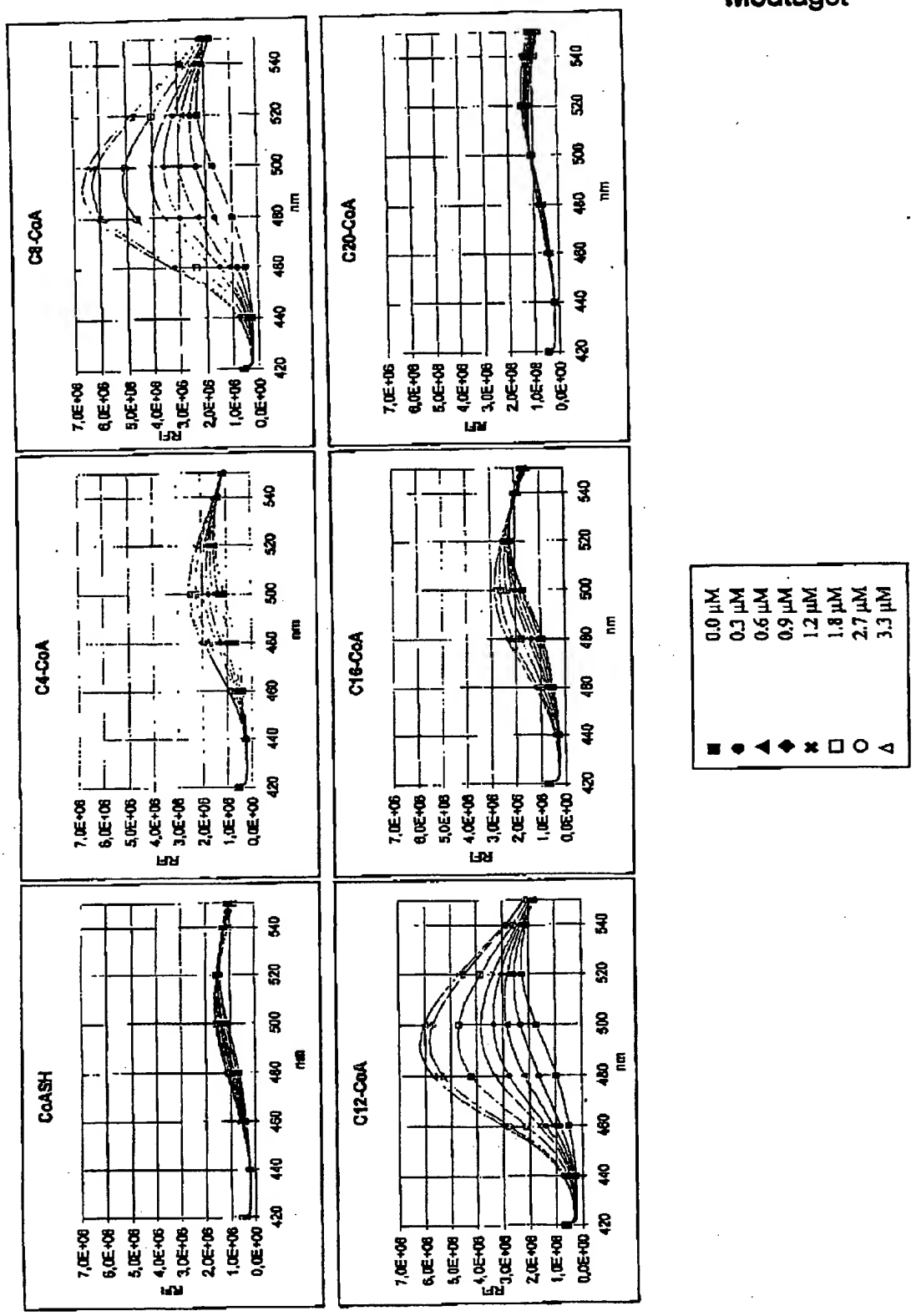
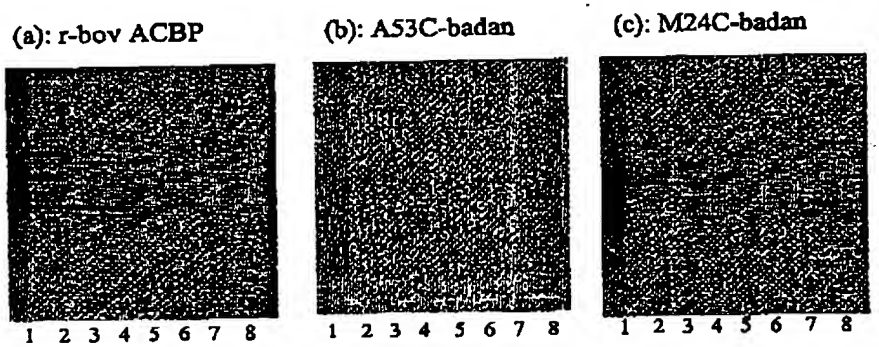


FIG. 3

Figure 4:



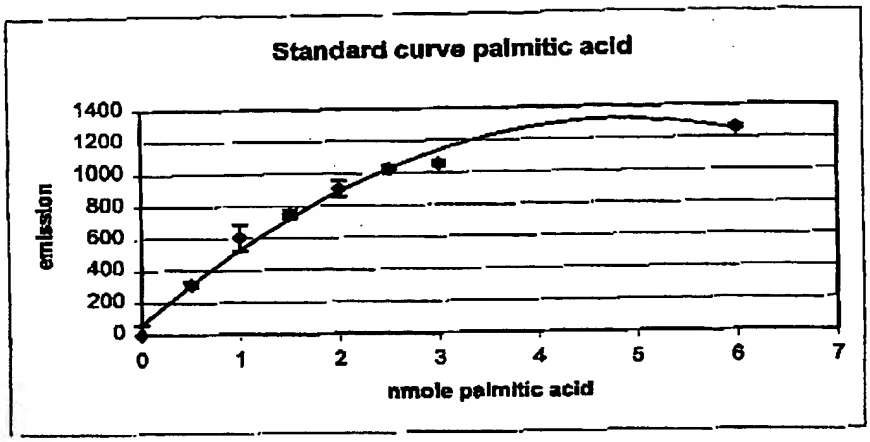

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Fig. 5a.

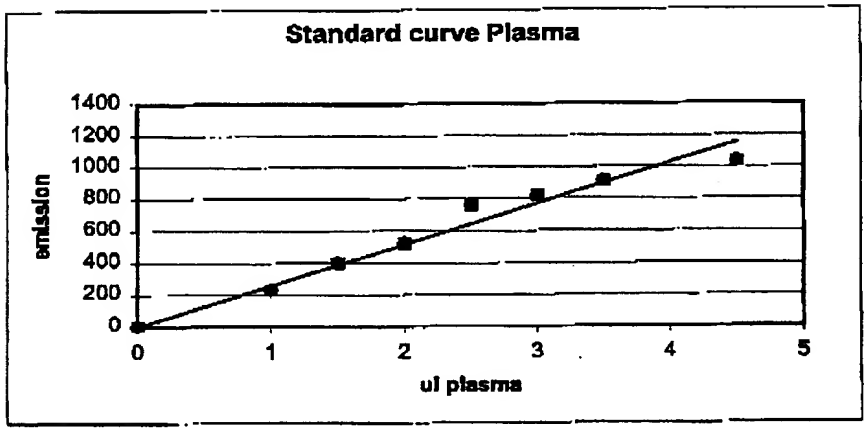
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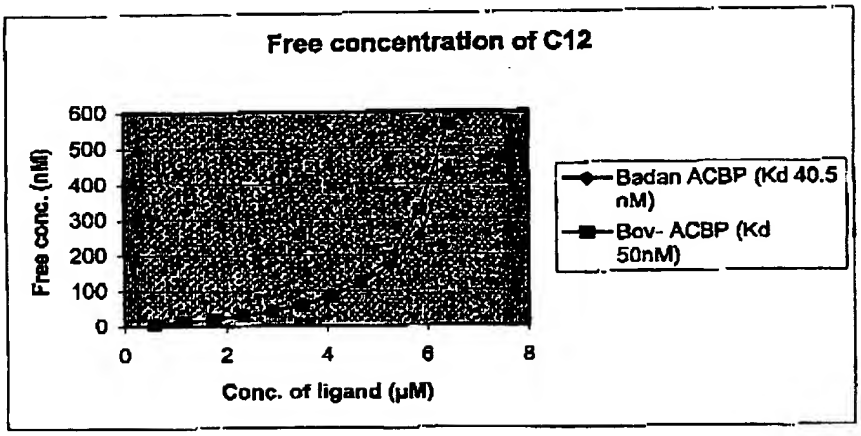
Fig. 5b.

Fig. 6



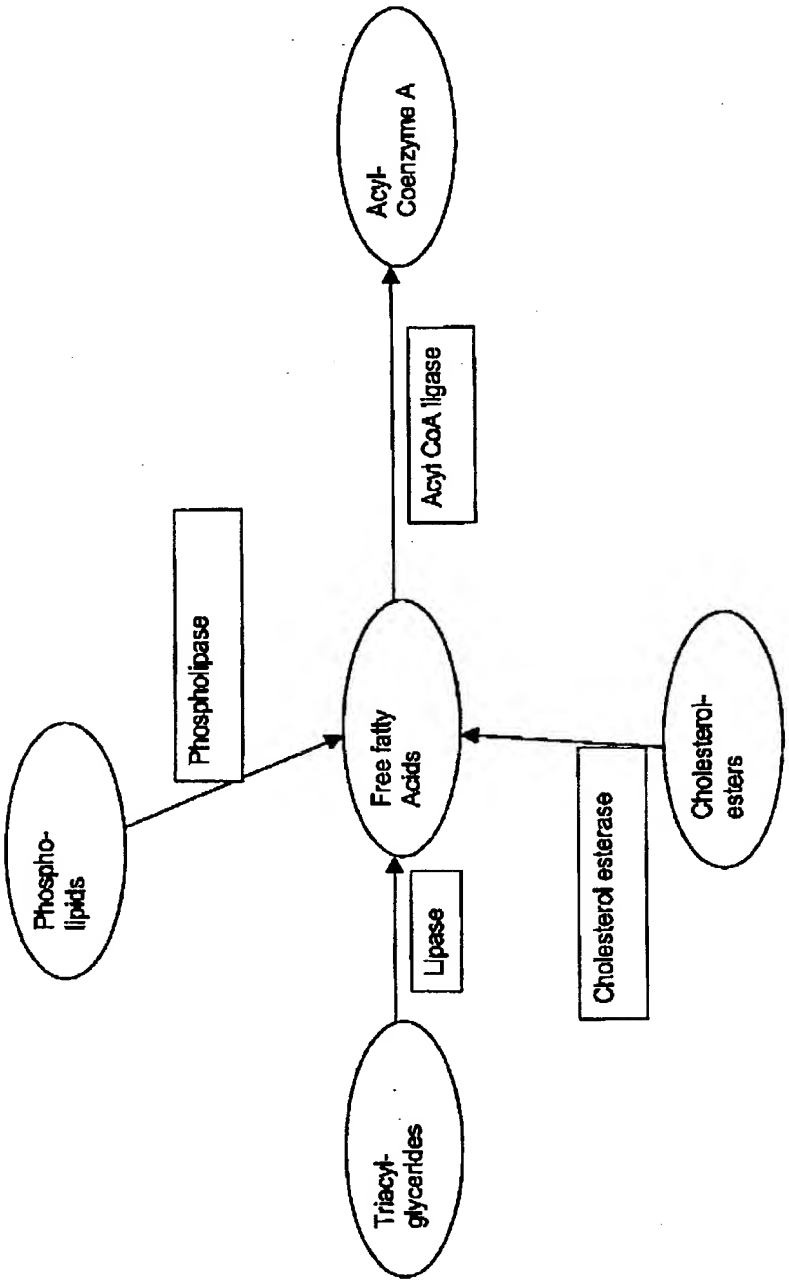


FIG. 7

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Gly Pro Val Ser Asp Gln Glu Lys Leu Leu Val Tyr Ser Tyr Tyr Lys
20 25 30

Gln Ala Thr Gln Gly Asp Cys Asn Ile Pro Ala Pro Pro Ala Thr Asp
35 40 45

Leu Lys Ala Lys Ala Lys Trp Glu Ala Trp Asn Val Glu Lys Gly Met
50 55 60

Ser Lys Met Asp Ala Met Arg Ile Tyr Ile Ala Lys Val Glu Glu Leu
65 70 75 80

Lys Lys Asn Glu Ala Gly
85

<210> 13
<211> 86
<212> PRT
<213> Dog

<400> 13
Ser Gln Ala Glu Phe Asp Lys Ala Ala Glu Asp Val Lys His Leu Lys
1 5 10 15

Thr Lys Pro Ala Asp Asp Glu Met Leu Tyr Ile Tyr Ser His Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Leu Leu Asp
35 40 45

Leu Arg Gly Lys Ala Lys Trp Asp Ala Trp Asn Gln Leu Lys Gly Thr
50 55 60

Ser Lys Glu Asp Ala Met Lys Ala Tyr Val Asn Lys Val Glu Asp Leu
65 70 75 80

Lys Lys Lys Tyr Gly Ile
85

<210> 14
<211> 86
<212> PRT
<213> Duck

<400> 14
Ala Glu Ala Ala Phe Gln Lys Ala Ala Glu Glu Val Lys Gln Leu Lys
1 5 10 15

Ser Gln Pro Ser Asp Gln Glu Met Leu Asp Val Tyr Ser His Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Val Asn Thr Asp Arg Pro Gly Met Leu Asp
35 40 45

Phe Lys Gly Lys Ala Lys Trp Asp Ala Trp Asn Ala Leu Lys Gly Met
50 55 60

Ser Lys Glu Asp Ala Met Lys Ala Tyr Val Ala Lys Val Glu Glu Leu
65 70 75 80

Lys Gly Lys Tyr Gly Ile
85

<210> 15
<211> 86
<212> PRT
<213> Duck, brain

<400> 15
His Gln Ala Asp Phe Asp Glu Ala Ala Glu Glu Val Lys Lys Leu Lys
1 5 10 15

Thr Arg Pro Thr Asp Glu Glu Leu Lys Glu Leu Tyr Gly Phe Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Ile Asn Ile Glu Cys Pro Gly Met Leu Asp
35 40 45

Leu Lys Gly Lys Ala Lys Trp Glu Ala Trp Asn Leu Lys Lys Gly Ile
50 55 60

Ser Lys Glu Asp Ala Met Asn Ala Tyr Ile Ser Lys Ala Lys Thr Met
65 70 75 80

Val Glu Lys Tyr Gly Ile

85

<210> 16
<211> 86
<212> PRT
<213> Frog, brain

<400> 16
Pro Gln Ala Asp Phe Asp Lys Ala Ala Gly Asp Val Lys Lys Leu Lys
1 5 10 15

Thr Lys Pro Thr Asp Asp Glu Leu Lys Glu Leu Tyr Gly Leu Tyr Lys
20 25 30

Gln Ser Thr Val Gly Asp Ile Asn Ile Glu Cys Pro Gly Met Leu Asp
35 40 45

Leu Lys Gly Lys Ala Lys Trp Asp Ala Trp Asn Leu Lys Lys Gly Leu
50 55 60

Ser Lys Glu Asp Ala Met Ser Ala Tyr Val Ser Lys Ala His Glu Leu
65 70 75 80

Ile Glu Lys Tyr Gly Leu
85

<210> 17
<211> 85
<212> PRT
<213> Fruitfly

<400> 17
Val Ser Glu Gln Phe Asn Ala Ala Ala Glu Lys Val Lys Ser Leu Thr
1 5 10 15

Lys Arg Pro Ser Asp Asp Glu Phe Leu Gln Leu Tyr Ala Leu Phe Lys
20 25 30

Gln Ala Ser Val Gly Asp Asn Asp Thr Ala Lys Pro Gly Leu Leu Asp
35 40 45

Leu Lys Gly Lys Ala Lys Trp Glu Ala Trp Asn Lys Gln Lys Gly Lys
50 55 60

Ser Ser Glu Ala Ala Gln Gln Glu Tyr Ile Thr Phe Val Glu Gly Leu
65 70 75 80

Val Ala Lys Tyr Ala
85

<210> 18
<211> 88
<212> PRT
<213> Hawkmoth

<400> 18
Leu Gln Glu Gln Phe Asp Gln Ala Ala Ser Asn Val Arg Asn Leu Lys
1 5 10 15

Ser Leu Pro Ser Asp Asn Asp Leu Leu Glu Leu Tyr Ala Leu Phe Lys
20 25 30

Gln Ala Ser Ala Gly Asp Ala Asp Pro Ala Asn Arg Pro Gly Leu Leu
35 40 45

Asp Leu Lys Gly Lys Ala Lys Phe Asp Ala Trp His Lys Lys Ala Gly
50 55 60

Leu Ser Lys Glu Asp Ala Gln Lys Ala Tyr Ile Ala Lys Val Glu Ser
65 70 75 80

Leu Ile Ala Ser Leu Gly Leu Gln
85

<210> 19
<211> 85
<212> PRT
<213> Lilly

<400> 19
Leu Lys Glu Glu Phe Glu Glu His Ala Val Lys Ala Lys Thr Leu Pro
1 5 10 15

Glu Ser Thr Ser Asn Glu Asn Lys Leu Ile Leu Tyr Gly Leu Tyr Lys
20 25 30

Gln Ser Thr Val Gly Pro Val Asp Thr Gly Arg Pro Gly Met Phe Ser
35 40 45

Pro Arg Glu Arg Ala Lys Trp Asp Ala Trp Lys Ala Val Glu Gly Lys
50 55 60

Ser Lys Glu Glu Ala Met Gly Asp Tyr Ile Thr Lys Val Lys Gln Leu
65 70 75 80

Leu Glu Glu Ser Ala
85

<210> 20
<211> 86
<212> PRT
<213> Homo sapiens

<400> 20
Ser Gln Ala Glu Phe Glu Lys Ala Ala Glu Glu Val Arg His Leu Lys
1 5 10 15

Thr Lys Pro Ser Asp Glu Glu Met Leu Phe Ile Tyr Gly His Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Met Leu Asp
35 40 45

Phe Thr Gly Lys Ala Lys Trp Asp Ala Trp Asn Glu Leu Lys Gly Thr
50 55 60

Ser Lys Glu Asp Ala Met Lys Ala Tyr Ile Asn Lys Val Glu Glu Leu
65 70 75 80

Lys Lys Lys Tyr Gly Ile
85

<210> 21
<211> 86
<212> PRT
<213> Mouse, testis

<400> 21
Ser Gln Val Glu Phe Glu Met Ala Cys Ala Ser Leu Lys Gln Leu Lys
1 5 10 15

Gly Pro Val Ser Asp Gln Glu Lys Leu Leu Val Tyr Ser Phe Tyr Lys
20 25 30

Gln Ala Thr Gln Gly Asp Cys Asn Ile Pro Val Pro Pro Ala Thr Asp
35 40 45

Val Arg Ala Lys Ala Lys Tyr Glu Ala Trp Met Val Asn Lys Gly Met

50 55 60

Ser Lys Met Asp Ala Met Arg Ile Tyr Ile Ala Lys Val Glu Glu Leu
65 70 75 80

Lys Lys Lys Glu Pro Cys
85

<210> 22
<211> 86
<212> PRT
<213> Mouse

<400> 22
Ser Gln Ala Glu Phe Asp Lys Ala Ala Glu Glu Val Lys Arg Leu Lys
1 5 10 15

Thr Gln Pro Thr Asp Glu Glu Met Leu Phe Ile Tyr Ser His Phe Lys
20 25 30

Gln Ala Thr Val Gly Asp Val Asn Thr Asp Arg Pro Gly Leu Leu Asp
35 40 45

Leu Lys Gly Lys Ala Lys Trp Asp Ser Trp Asn Lys Leu Lys Gly Thr
50 55 60

Ser Lys Glu Ser Ala Met Lys Thr Tyr Val Glu Lys Val Asp Glu Leu
65 70 75 80

Lys Lys Lys Tyr Gly Ile
85

<210> 23
<211> 86
<212> PRT
<213> Pig

<400> 23
Ser Gln Ala Glu Phe Glu Lys Ala Ala Glu Glu Val Lys Asn Leu Lys
1 5 10 15

Thr Lys Pro Ala Asp Asp Glu Met Leu Phe Ile Tyr Ser His Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Ile Leu Asp
35 40 45

Leu Lys Gly Lys Ala Lys Trp Asp Ala Trp Asn Gly Leu Lys Gly Thr
50 55 60

Ser Lys Glu Asp Ala Met Lys Ala Tyr Ile Asn Lys Val Glu Glu Leu
65 70 75 80

Lys Lys Lys Tyr Gly Ile
85

<210> 24
<211> 86
<212> PRT
<213> Brassica napus

<400> 24
Leu Lys Glu Asp Phe Glu Glu His Ala Glu Lys Val Lys Lys Leu Thr
1 5 10 15

Ala Ser Pro Ser Asn Glu Asp Leu Leu Ile Leu Tyr Gly Leu Tyr Lys
20 25 30

Gln Ala Thr Val Gly Pro Val Thr Thr Ser Arg Pro Gly Met Phe Ser
35 40 45

Met Lys Glu Arg Ala Lys Trp Asp Ala Trp Lys Ala Val Glu Gly Lys
50 55 60

Ser Thr Asp Glu Ala Met Ser Asp Tyr Ile Thr Lys Val Lys Gln Leu
65 70 75 80

Leu Glu Ala Glu Ala Ser
85

<210> 25
<211> 86
<212> PRT
<213> Rat, testis

<400> 25
Ser Gln Val Glu Phe Glu Met Ala Cys Ala Ser Leu Lys Gln Leu Lys
1 5 10 15

Gly Pro Leu Ser Asp Gln Glu Lys Met Leu Val Tyr Ser Phe Tyr Lys
20 25 30

Gln Ala Thr Gln Gly Asp Cys Asn Ile Pro Val Pro Pro Ala Thr Asp
35 40 45

Val Lys Ala Lys Ala Lys Trp Glu Ala Trp Met Val Asn Lys Gly Met
50 55 60

Ser Lys Met Asp Ala Met Arg Ile Tyr Ile Ala Lys Val Glu Glu Leu
65 70 75 80

Lys Lys Asn Glu Thr Cys
85

<210> 26
<211> 86
<212> PRT
<213> Rat

<400> 26
Ser Gln Ala Asp Phe Asp Lys Ala Ala Glu Glu Val Lys Arg Leu Lys
1 5 10 15

Thr Gln Pro Thr Asp Glu Glu Met Leu Phe Ile Tyr Ser His Phe Lys
20 25 30

Gln Ala Thr Val Gly Asp Val Asn Thr Asp Arg Pro Gly Leu Leu Asp
35 40 45

Leu Lys Gly Lys Ala Lys Trp Asp Ser Trp Asn Lys Leu Lys Gly Thr
50 55 60

Ser Lys Glu Asn Ala Met Lys Thr Tyr Val Glu Lys Val Glu Glu Leu
65 70 75 80

Lys Lys Lys Tyr Gly Ile
85

<210> 27
<211> 86
<212> PRT
<213> Turtle

<400> 27
Ser Gln Ala Glu Phe Asp Lys Ala Ala Glu Glu Val Lys Gln Leu Lys
1 5 10 15

Ser Gln Pro Thr Asp Glu Glu Met Leu Tyr Ile Tyr Ser His Phe Lys

20 25 30

Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Phe Leu Asp
35 40 45

Phe Lys Gly Lys Ala Lys Trp Asp Ala Trp Asp Ala Leu Lys Gly Met
50 55 60

Ala Lys Glu Glu Ala Met Lys Ala Tyr Ile Ala Lys Val Glu Glu Leu
65 70 75 80

Lys Gly Lys Tyr Gly Ile
85

<210> 28
<211> 86
<212> PRT
<213> Saccharomyces cerevisiae

<400> 28
Val Ser Gln Leu Phe Glu Glu Lys Ala Lys Ala Val Asn Glu Leu Pro
1 5 10 15

Thr Lys Pro Ser Thr Asp Glu Leu Leu Glu Leu Tyr Ala Leu Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Asn Asp Lys Glu Lys Pro Gly Ile Phe Asn
35 40 45

Met Lys Asp Arg Tyr Lys Trp Glu Ala Trp Glu Asn Leu Lys Gly Lys
50 55 60

Ser Gln Glu Asp Ala Glu Lys Glu Tyr Ile Ala Leu Val Asp Gln Leu
65 70 75 80

Ile Ala Lys Tyr Ser Ser
85

<210> 29
<211> 86
<212> PRT
<213> Saccharomyces monoasensis

<400> 29
Val Ser Gln Leu Phe Glu Glu Lys Ala Lys Ala Val Asn Glu Leu Pro
1 5 10 15

15

Thr Lys Pro Ser Thr Asp Glu Leu Leu Glu Leu Tyr Gly Leu Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Asn Asp Lys Glu Lys Pro Gly Ile Phe Asn
35 40 45

Met Lys Asp Arg Tyr Lys Trp Glu Ala Trp Glu Asp Leu Lys Gly Lys
50 55 60

Ser Gln Glu Asp Ala Glu Lys Glu Tyr Ile Ala Tyr Val Asp Asn Leu
65 70 75 80

Ile Ala Lys Tyr Ser Ser
85

<210> 30
<211> 86
<212> PRT
<213> Cow liver

<400> 30
Ser Gln Ala Glu Phe Asp Lys Ala Ala Glu Glu Val Lys His Leu Lys
1 5 10 15

Thr Lys Pro Ala Asp Glu Glu Met Leu Phe Ile Tyr Ser His Tyr Lys
20 25 30

Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Met Leu Asp
35 40 45

Phe Lys Gly Lys Ala Lys Trp Asp Ala Trp Asn Glu Leu Lys Gly Thr
50 55 60

Ser Lys Glu Asp Ala Met Lys Ala Tyr Ile Asp Lys Val Glu Glu Leu
65 70 75 80

Lys Lys Lys Tyr Gly Ile
85